INVESTIGATION OF ANTIMICROBIAL RESISTANCE AND ANTIMICROBIAL USE IN WESTERN CANADIAN COW-CALF HERDS

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By

Sheryl Pamela Gow

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ABSTRACT

This thesis summarizes an investigation of antimicrobial resistance (AMR) and antimicrobial use (AMU) in cow-calf herds. The specific objectives of this project were to describe common reasons for treatment and the types of antimicrobials used in cow-calf herds, to describe the frequency of AMR in generic fecal *Escherichia coli* isolated from various age groups commonly found on cow-calf farms, to determine risk factors associated with the occurrence of AMR, and finally to investigate the underlying molecular mechanisms of AMR in cow-calf herds. At least 86% of the herds treated one or more calves or cows during the study period; however, the overall proportion of both calves and cows reported as treated was less than 14% for calves and 3% for cows. The majority of antimicrobials reported as used in cow-calf operations were for individual therapeutic use rather than prophylaxis, metaphylaxis, or growth promotion. Injectable formulations were the most commonly reported method of antimicrobial administration on cow-calf farms. Cow-calf herds in Wetern Canada are not a significant reservoir for resistance to antimicrobials classified as very important to human medicine such as ciprofloxacin and ceftiofur. The three most common resistances detected were to tetracycline, sulphamethoxazole, and streptomycin regardless of age group. Young calves sampled in the spring of the year were more likely to be shedding AMR *E. coli* than older calves sampled in the fall of the year or than cows sampled in the spring of the year. The cow-calf pair relationship was not an important factor in transfer of AMR from the individual cow to her calf, but the presence of AMR in the general cow herd was associated with AMR in the calf population. The potential importance of co-selection for AMR at the molecular level was demonstrated by both the risk factor

analysis and the molecular work. Phenotypic resistance to streptomycin, tetracycline, and sulphamethoxazole were each associated with the presence of resistance genes from all six families of antimicrobials examined in this study. Several statistically significant associations were also detected between the resistance genes considered. No significant associations were detected between any of the AMR phenotypes or genotypes and the STEC virulence factors *stx*1, *stx*2 and *eae*.

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I could pursue a degree in epidemiology. It has been a long, arduous journey and I am grateful to you all for making it with me.

DEDICATION

I would like to dedicate this work to my husband and family who supported me throughout this process and to my colleagues, co-workers and friends at the Public Health Agency of Canada, especially the CIPARS on farm working group who ensured that I had the time and the energy to complete this thesis.

ORIGINAL CONTRIBUTION

The field work for this thesis was completed as an extension to a larger project entitled: “Western Canada Study of Animal Health Effects Associated with Exposures to Emissions from Oil and Natural Gas Field Facilities”, commonly referred to as the Western Canada Beef Productivity Study (WCBPS). The design and implementation of the WCBPS were established prior to the initiation of this Ph.D. program. I was one of six project veterinarians employed by the WCBPS to visit study herds and collect samples and data. Project veterinarians were also responsible for all data entry.

My contribution to the intellectual property for the AMR and AMU projects described in this thesis was in collaborating on the design, coordinating the field management, and completing the data analysis. I coordinated fecal sample collection, submitted all samples to the laboratory, entered data pertaining to samples, entered animal and AMU data pertaining to the ten herds for which I was directly responsible, and analyzed all data pertaining to this thesis.

I also contributed to preparing and submitting the grant proposals to obtain the funding for fecal sample collection, processing, and laboratory analysis. Additional laboratory support and funding was provided by the Agri-Food Laboratories Branch, Food Safety Division, Alberta Agriculture and Food, Edmonton, Alberta, Canada and the Laboratory for Foodborne Zoonoses, Public Health Agency of Canada, Guelph, Ontario, Canada. Fecal samples were cultured by Prairie Diagnostic Services (PDS),

Western College of Veterinary Medicine Saskatoon, Saskatchewan. Susceptibility testing was completed by Alberta Agriculture and Food. Antimicrobial resistance gene molecular work was performed in Département de Pathologie et Microbiologie, Faculté de Médecine Vétérinaire, Université de Montréal, Saint-Hyacinthe, Québec, Canada. Virulence testing was also performed by PDS, Saskatoon, Saskatchewan.

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LIST OF ABBREVIATIONS

Aac	Acetyltransferases- Aminoglycoside resistance gene
aad(ant)	Adenlytransferases- Aminoglycoside resistance gene
ADD	Animal daily dose
A3C	Ampicillin, cefoxitin, ceftiofur, cephalothin
ACSSuT	Ampicillin, chloramphenicol, streptomycin, sulphamethoxazole, tetracycline
ACKSSuT	Ampicillin, chloramphenicol, kanamycin, streptomycin, sulphamethoxazole, tetracycline
AKSSuT	Ampicillin, kanamycin, streptomycin, sulphamethoxazole, tetracycline
AMR	Antimicrobial resistance
AMU	Antimicrobial use
Aph	Adenylphosphotransferases- Aminoglycoside resistance gene
bla_{TEM}	Beta-lactam resistance gene
bla_{SHV}	Beta-lactam resistance gene
$bla_{cmy\text{-}2}$	Beta-lactam resistance gene
BVDV	Bovine virial diarrhea virus
cat	Chloramphenicol resistance gene
CIPARS	Canadian Integrated Program for Antimicrobial Resistance Surveillance
CI	Confidence interval
clm	Chloramphenicol resistance gene
DDD	Defined daily doses

Dhfr	Trimethoprim resistant dihydrofolate reductase gene
DNA	Deoxyribonucleic acid
eae	Enterocyte attaching and effacing *E. coli* virulence factor
E. coli	*Escherichia coli*
*flo*R	Florphenicol resistance gene
GEE	Generalized estimating equations
HC	Hemorrhagic colitis
HUS	Hemolytic uremic syndrome
IBR	Infectious bovine rhinotracheitis
IKC	Infectious bovine keratoconjunctivitis
IQR	Inter-quartile range
LB	Luria-Bertani broth
LEE	Locus for enterocyte effacement
MIC	Minimum inhibitory concentration
Multi-AMR	Multiple antimicrobial resistance
NAHMS	National Animal Health Monitoring System
NARMS	National Antimicrobial Resistance Monitoring System
N	Number of herds
n	Number of animals
NCCLS	National Committee on Clinical Laboratory Standards
OR	Odds ratio
PCR	Polymerase chain reaction
PQL	Penalized quasi-likelihood

S. ohio	*Salmonella ohio*
STEC	Shiga-toxigenic *E. coli*
S. typhimurium	*Salmonella typhimurium*
stx	Shiga toxin
sul	Sulphonamide resistance gene
tet	Tetracycline resistance gene
TSI	Triple sugar iron
μg/mL	Microgram/milliliter
USDA	United States Department of Agriculture
VDD	Veterinary Drug Directorate
*vt*1	Verotoxins
+	Positive

CHAPTER 1
INTRODUCTION

1.1 Background

Antimicrobial resistance (AMR) is a growing public health concern. The spread of AMR and the appearance of multiple antimicrobial resistant pathogenic bacteria have been recognized by the World Health Organization (WHO) as serious problems that can complicate medical treatment of bacterial infections (WHO, 2001). The increase in the number of antimicrobial resistant pathogens in human medicine has raised both public and scientific interest, and some of this concern has focussed on antimicrobial use (AMU) in livestock production.

Most AMR in human pathogens is attributable to the selection pressure from AMU in people (Thompson, 2000). However, the volume of antimicrobials used in food animal production has led to concerns in the public, regulatory and scientific arenas that AMU in food animals could contribute to the AMR problem by creating a reservoir of resistant bacteria (Bailar and Travers, 2002, O'Connor et al., 2002; Schroeder et al., 2002). For human health, the transfer of such resistance to zoonotic enteropathogens is of primary interest, but the transfer of resistance to animal pathogens and the associated subsequent loss of therapeutic options for veterinary medicine is also an important concern.

At this time, AMR is not a major clinical problem in veterinary medicine in Canada; however, the impact of AMR in human medicine and the occurrence of AMR as a veterinary problem in other parts of the world indicate that this is a real possibility (McEwen, 2002). The use of antimicrobial drugs in animal agriculture is essential for maintaining and improving animal health and welfare through disease treatment, increasing carcass quality, and enhancing the economic efficiency of growth and production. If the livestock industry loses efficacious antimicrobials because of resistance development or limited access because of tighter regulations, the consequences and costs to the industry would be substantial.

1.2 Investigative approach

Although there is a growing amount of literature on AMR, no information is currently available regarding AMR or AMU in cow-calf herds in western Canada. The cow-calf industry is a vital and important part of the agricultural economy in all parts of Canada, but particularly in Saskatchewan and Alberta. These two provinces are home to more than 65% of the beef cow, breeding heifer and calf populations in Canada (Statistics Canada, Accessed July 25, 2006; http://www.40.statcan.ca/101/cst01/prim50a.htm). A better understanding of AMR and AMU patterns in this population is essential to develop a baseline of data to determine the need for future monitoring in the Canadian cow-calf industry.

This investigation was undertaken to provide initial data on AMR and AMU in cow-calf herds in order to describe common reasons for treatment and the types of antimicrobials used on cow-calf farms, to describe the amount of AMR in various age groups commonly found on cow-calf farms, to determine risk factors associated with AMR development, and finally to investigate the underlying molecular mechanisms of AMR in cow-calf herds.

This project represented a collaborative research initiative undertaken to address the presence of and risk factors for AMR in western Canadian cow-calf herds. The Western College of Veterinary Medicine, the Public Health Agency of Canada, and Alberta Agriculture worked together to address this important question. The primary hypothesis of this dissertation was that AMR in fecal generic *Escherichia coli* isolates collected from cow-calf herds would be relatively less prevalent than from other food-animal species because these animals are extensively managed as compared with most other livestock commodities. A secondary hypothesis was that although use and resistance would likely be associated with each other, routine AMU would be relatively uncommon in most cow-calf operations. The final hypothesis examined in this study was that the statistical associations between AMR genes present in the *E.coli* isolates would likely support evidence of co-selection of unrelated resistance genes and virulence factors of interest.

The specific objectives of this investigation were;

1. To describe the frequency of treatment with antimicrobials, common reasons for AMU, and the types of antimicrobials used in western Canadian cow-calf herds.
2. To describe AMR in calves from western Canadian cow-calf herds in the spring and fall of 2002 using commensal *E. coli* as an indicator organism.
3. To describe AMR in cows and cow-calf pairs from western Canadian beef herds in the spring using commensal *E. coli* as an indicator organism.
4. To investigate farm level management practices associated with AMR in commensal *E. coli* isolates collected from calves during the 2002 calving season on beef herds in western Canada.
5. To measure the associations between antimicrobial resistant phenotypes and resistance genes in commensal *E. coli* isolates obtained from cattle in cow-calf herds to understand the potential for co-selection and genetic linkages.
6. To describe the associations between genetic determinants of antimicrobial resistance in commensal *E. coli* isolates obtained from cattle in cow-calf herds to understand the potential for coselection and genetic linkages.
7. To investigate whether either AMR phenotype or genotype are associated with the presence of the virulence genes *stx*1, *stx*2 and *eae* in commensal *E. coli* isolates from cattle in cow-calf herds.

The participating cow-calf producers from across Alberta and Saskatchewan were also involved in a multifaceted survey of risk factors affecting cattle productivity and health. Private veterinary clinics were approached and asked to participate. Within each practice herds were identified and enrolled based on selection criteria which considered

factors such as herd size, animal identification, existing calving records, animal handling facilities sufficient for pregnancy testing and bull evaluation, and a relationship with a local veterinary clinic. Only herds using a winter/spring calving season were enrolled in the study. Participating herds were visited regularly by one of six study veterinarians to collect samples and data, and to monitor the quality and consistency of on-farm records. Data on AMU were collected using both individual animal treatment records and a standardized questionnaire.

In a first step of this study (Chapter 2), the literature on AMR and AMU were reviewed and gaps in the existing literature were identified as they relate to the objectives of this thesis. Reported reasons for antimicrobial treatment and potential risk factors for treatment in both cows and calves were examined in Chapter 3. The prevalence of AMR and the importance of individual animal determinants of AMR status was then described in Chapters 4 and 5 by investigating the extent of AMR in young calves, calves at weaning, and cows. The role of the cow-calf pair relationship in the transfer of resistance was also explored in Chapter 5. In Chapter 6, the importance of herd-level risk factors associated with AMR in calves born and sampled in the spring of 2002 was investigated. The molecular aspects of AMR were then considered in a sub-set of isolates. The association between AMR phenotype and genotype was initially assessed (Chapter 7) and then the relationship between AMR genes was examined (Chapter 8). The genetic aspect of this project provided an opportunity to explore the potential for co-selection of AMR determinants in these isolates. As a final part of the molecular investigation, Chapter 9 examined the virulence factors *stx*1, *stx*2 and *eae* and

their relationship to either the AMR phenotype or genotype because of the potential public health impact. Finally, Chapter 10 summarizes the new information generated by and the limitations of this field study, it also provides suggestions for further research.

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CHAPTER 2
LITERATURE REVIEW

2.1. Introduction

Antimicrobial resistance (AMR) is an important issue facing both human and veterinary medicine. The primary concern in veterinary medicine is not treatment failure as a result of AMR, but that the use of antimicrobials in food animal production could promote the development of resistance in people. The debate about the role of agriculture in the distribution and magnitude of AMR in people has been on going since before the release of the Swann report in 1969 (Prescott, 2000).

There is a tremendous amount of literature on AMR and antimicrobial use (AMU) in both human and veterinary medicine. This is not intended to be an exhaustive review of these subjects, but rather to provide readers that are not intimately involved in this area of research with sufficient background to understand the following thesis and to recognize that AMR is a complex issue which still requires more research. The issues summarized included: detection methods for AMR, how AMR is transferred, how some key antimicrobials exert their effect and how bacteria combat these antimicrobials, AMR and virulence factors, the challenges of collecting and reporting AMU information, and AMU/AMR in livestock with a particular focus on the beef cattle

industry. The review will focus on AMR in fecal *Escherichia coli* (*E.coli*) in cattle unless otherwise stated.

Standard search engines such as Agricola, CAB abstracts, and Ovid MEDLINE as well as non-scientific search engines including 'google' were used for literature searches. Search terms included, but were not limited to, combinations of: antimicrobial, antibiotic, use, exposure, treatment, susceptibility, resistance, *Escherichia coli,* bovine, cattle, cow-calf, herd, and farm. Cited references were examined for additional resources. An English language restriction was used.

2.2. Antimicrobial resistance (AMR)

Antimicrobial resistance is a form of natural selection and is an expected phenomenon (McDermott et al., 2002). Resistant micro-organisms were present long before the introduction of antimicrobials, and resistance was probably a defense mechanism used by antibiotic-producing organisms to protect themselves (Smith, 1967, Dancer et al., 1997). Therefore in the presence of an antimicrobial, the bacteria that possess an effective resistance trait will survive and those that do not will be eliminated. In an environment with long-term antimicrobial exposure, the proportion of resistant bacteria will increase over time (McDermott et al., 2002).

Levy (1998) described five basic principles of AMR. First, given sufficient time and use of an antimicrobial, resistance will develop in a susceptible organism. Resistant organisms have been identified for all types of antimicrobials. Second, AMR is

progressive and can be monitored by studying changes in minimum inhibitory concentrations. Third, bacteria resistant to one antimicrobial are more apt to become resistant to others. Fourth, once resistance appears, it is likely to decline slowly, if at all. Fifth, the use of antimicrobials in one individual affects others in the surrounding environment.

2.2.1. Definitions

To ensure clarity, definitions of the key terminology used throughout this project have been provided. An antibiotic is a substance that is produced by a microorganism and at low concentrations inhibits or kills other microorganisms (Prescott, 2000, Guardabassi and Courvalin, 2006). An antimicrobial includes any substance of natural, semisynthethic, or synthethetic origin that kills or inhibits the growth of a microorganism, but causes little or no damage to the host (Prescott, 2000, Guardabassi and Courvalin, 2006). Although the two terms differ in their precise definition, antimicrobial is often used synonymously with antibiotic (Prescott, 2000). Antimicrobial was the term used throughout this thesis.

Resistance can be a result of an intrinsic mechanism that prevents the bacteria from being destroyed by an antimicrobial, or it can be acquired through chromosomal mutation or the exchange of genetic material. Bacteria that are intrinsically resistant lack the structural or functional cellular mechanisms that are required for the antimicrobial to act (Prescott, 2000, Guardabassi and Courvalin, 2006). Intrinsic resistance is a genus or species specific property of bacteria (Schwarz et al., 2006).

Acquired resistance can develop and be transferred in susceptible organisms as a result of mutation, horizontal acquisition of foreign genetic material, or a combination of these processes (Catry et al., 2003, Guardabassi and Courvalin, 2006). The focus of this review and thesis is on acquired resistance. Additional information on the mechanisms of acquired resistance development and spread are provided later on in this review.

2.3. Susceptibility testing

Susceptibility testing is used to guide therapy and to generate surveillance data (Potz et al., 2004). Susceptibility is determined by growth inhibition and not the killing of bacterium (Walker, 2000). The results can be reported quantitatively or qualitatively. Qualitative results are reported as susceptible, intermediate, or resistant, while quantitative results provide a minimum inhibitory concentration (MIC) in μg/ml or mg/ml. The MIC is defined as the lowest concentration of drug required to inhibit growth of an organism using a standardized test (Jorgensen, 2004) and can be monitored to determine if a population is shifting towards increasing resistance (Walker, 2000).

Practitioners often require a clinically relevant category derived from applying interpretative breakpoints to the MIC information (Craig, 1993, MacGowan and Wise, 2001). Interpretive breakpoints allow for susceptible, intermediate, and resistant categorization of isolates. The break point for susceptibility is the recommended dosage of an antimicrobial that inhibits the bacterium's growth (Walker, 2000). Breakpoints for resistance represent concentrations that cannot be achieved by normal dosing, and

intermediate breakpoints are those which fall between susceptible and resistant (Walker, 2000).

Laboratory assessment of susceptibility and resistance is not necessarily equivalent to clinical susceptibility and resistance. Clinically (or in vivo) a strain is considered resistant if it survives therapy (Guardabassi and Courvalin, 2006). Clinical resistance can vary depending on the dosage, mode of drug administration, distribution of the drug, and the immune status of the patient (Guardabassi and Courvalin, 2006). Clinical breakpoints indicate the MIC that will reflect the probability of treatment success given a specified dosing schedule (Mouton, 2002). Clinical breakpoints are set not only based on the MICs, but also *in vivo* parameters such as pharmacokinetics and pharmacodynamics of the drug as well as with correlation of the MICs with the clinical outcome (Guardabassi and Courvalin, 2006). Factors such as bacterial distribution in the host, sub-MIC effects, postantibiotic effects, protein binding, and variations in drug concentration in the blood can all affect *in vivo* susceptibility (Jorgensen, 2004) and determination of the clinical breakpoint. An excellent overview of approaches that can be used to calculate clinical breakpoints is provided by Mouton (2002).

Breakpoints can also be considered from a microbiological (*in vitro*) rather than a clinical point of view. Microbiological breakpoints are based on MICs for a bacterial species with resistance at the higher MICs when compared to the distribution of the normal susceptible population (Guardabassi and Courvalin, 2006). Microbiological resistance is determined by comparison of two or more strains under identical

conditions (Guardabassi and Courvalin, 2006). These breakpoints are useful for surveillance and for identifying emerging resistance (Guardabassi and Courvalin, 2006).

Microbiological breakpoints are used to detect organisms that do no belong to the natural bacterial population. These organisms have acquired resistance and may represent an emerging resistant strain (Mouton, 2002). The microbiological breakpoint criteria do not consider drug pharmacokinetic properties in individual patients (Dudley and Ambrose, 2000, Mouton, 2002).

Breakpoints are generally derived from human isolates (Walker, 2000). The pharmacokinetic data collected from human populations may differ significantly from that derived from animals; therefore, what may be an appropriate breakpoint for human isolates may not be the same for animal isolates. Since human breakpoints do not reliably predict clinical outcomes when applied to veterinary pathogens, the National Committee for Clinical Laboratory Standards (NCCLS) have developed a veterinary specific antimicrobial susceptibility criteria (NCCLS, 2000).

There are other challenges associated with the reporting of breakpoints and AMR. Resistance can only be assessed by comparing the strains of the same species or genus (Guardabassi and Courvalin, 2006). For example, ampicillin has an MIC breakpoint of 128 μg/ml for *E. coli* sp. but for *Streptococcus agalacttiae* the MIC is 0.12 μg/ml (Prince and Neu, 1983). Breakpoints may also vary between countries (MacGowan and Wise, 2001, Mouton, 2002, Jorgensen, 2004) depending on the agency setting the

breakpoints and the methodologies used. Therefore, when comparing susceptibility results between different organism and countries one must keep in mind what the susceptible, intermediate, and resistant breakpoints are for each respective organism or country. Despite these limitations, susceptibility breakpoints can provide a reference for clinical efficacy (Jorgensen, 2004) and for surveillance purposes.

2.3.1. Phenotype susceptibility testing methods

The primary methods used for susceptibility testing are agar disc diffusion, broth microdilution, agar dilution, broth macrodilution, and E-test. Since agar diffusion and broth microdilution are the two principal methodologies used in veterinary medicine (Brooks et al., 2003), this discussion will focus on these tests and some of their advantages and disadvantages.

Agar disc diffusion is based on diffusion of an antimicrobial agent from a commercially prepared disc placed on an agar surface inoculated with a standardized growth medium that has been seeded with approximately 1.0×10^8 colony forming units of pure culture (Prescott, 2000). At the same time that the inoculum is growing, the antimicrobial agent is diffusing from the disc. If the organism is susceptible to the antimicrobial, a zone of growth inhibition is created around the disc. The larger the zone of inhibition, the more susceptible the organism is to the antimicrobial.

Agar disc diffusion techniques provide qualitative data, are flexible and low cost. However, the results of disc diffusion will vary unless the inoculum density, the agar

thickness and the incubation are carefully controlled (Potz et al., 2004). Veterinary specific antimicrobial disks are available for antimicrobials such as ceftiofur, enrofloxacin, and tilmicosin (Watts and Lindeman, 2006). Agar disc diffusion breakpoints are derived from the relationship between the zones of inhibition to the MIC (Craig, 2000).

Agar dilution is the gold standard, but it and broth macrodilution are often too cumbersome for routine use and so are often replaced with broth microdilution (Walker, 2000). Broth microdilution involves using a microplate that contains antimicrobial agents of known concentration in progressive two fold dilutions that encompass similar concentrations to those obtained in serum and tissue at recommended doses (Walker, 2000). To perform broth microdilution, a bacterial suspension is made from an overnight culture of a single randomly selected isolate, diluted to turbidity comparable to a 0.5 McFarland standard (Walker, 2000). This is further diluted so that the final concentration of bacteria per well is 5×10^4 colony forming units (Walker, 2000). The plates are then incubated for 16-20 hours (Walker, 2000). The minimum inhibitory concentration is recorded as the lowest concentration of antimicrobial that completely inhibits growth.

Broth microdilution and agar dilution both provide MICs by exposing the organism to a series of twofold log dilutions of the antimicrobial of interest (Jorgensen, 2004). These are the preferred methods of surveillance systems (Watts and Lindeman, 2006)

because they can demonstrate trends in MICs over time. Broth microdilution can be highly automated and, therefore, is capable of handling large volumes of samples.

The disadvantage is that broth microdilution utilizes MIC panels that are often inflexible as to the dilution and the antimicrobials available on a specified panel. Custom plates can be designed, but they are often cost prohibitive for many laboratories. Another limitation is that because only a few (1-10) isolates/sample are selected for testing and MICs may fail to identify minority strains present in a complex polyclonal population unless a large number of isolates are investigated (Hedges et al., 1977, Humphrey et al., 2002). Also, under selective pressure of antimicrobial treatment, such minority species, if expressing a suitable phenotype, may be capable of dominating the microflora and potentially giving rise to sub-clinical or even clinical disease (Linton et al., 1978). As such random isolate selection may fail to fully describe the clinical importance AMR of any given bacterial population.

2.4. Molecular aspects of antimicrobial resistance

The phenotype provides an indication of the susceptibility of the organism and its potential impact clinically, but it does not indicate the genes present or the underlying mechanism for resistance. The genotype on the other hand does not imply whether a strain is sensitive or not to a specific antimicrobial, but it demonstrates the diversity and distribution of resistance genes (Aarts et al., 2006). Because each methodology provides different information, considering both the phenotype and genotype will provide a more complete understanding AMR.

Bacteria are very proficient at sharing the genetic information necessary to survive in the presence of antimicrobials (McDermot et al., 2002). The ability to readily exchange genes increases the possibility for the spread of AMR determinants from commensal organisms to pathogens (Salyers and Cuevas, 1997). Even transient passage of an ingested resistant organism through the intestinal tract can result in the transfer of resistant genes to resident microflora, which can then serve as a reservoir for pathogenic bacteria (McDermott et al., 2002).

2.4.1. How bacteria acquire resistance

There are two major ways that susceptible bacteria acquire AMR, mutation or horizontal gene transfer.

2.4.1.1. Mutation

Mutation is the spontaneous change in the genome from susceptible to resistant, usually during replication (Catry et al., 2003). Chromosomal mutations often result in structural changes to the bacterial cell wall and subsequent resistance development (Prescott, 2000, Guardabassi and Courvalin, 2006). They may lead to dramatic resistance development or to slower more gradual resistance development depending on the antimicrobial agent affected (Prescott, 2000, Guardabassi and Courvalin, 2006).

Mutants may be disadvantaged compared to the parent and, therefore, be less able to survive in the population in the absence of the selective pressure of an antimicrobial (Prescott, 2000). Alternatively, mutants maybe as viable as the parent and may persist in the population with or without selective pressure from AMU (Prescott, 2000). Mutational events happen at high frequencies for drugs such as streptomycin, nalidixic acid, and rifampin; whereas, the mutation frequency to erythromycin are lower and almost non-existent for vancomycin and polymixin B (Prescott, 2000).

2.4.1.2. Horizontal transfer of resistance

The horizontal transfer of resistance genes from donor to recipient bacteria is a second method through which bacteria can acquire resistance. The three primary methods for horizontal resistance gene transfer are transformation, transduction, and conjugation (Schwarz and Chaslus Dancla, 2001).

Transformation is the uptake of naked bacterial DNA from the environment by an acceptor bacteria (Prescott, 2000, Schwarz and Chaslus-Dancla, 2001). It is a critically important method of gene transfer (Prescott, 2000) *in vitro* but less important *in vivo* (Schwarz et al., 2006). Transformation generally occurs between closely related genera and may result in gene recombination producing new forms of resistance genes (Prescott, 2000). This method of resistance transfer is particularly important in bacteria species such as *Streptococcus* spp. and *Neisseria* spp. that have a high frequency of natural transformation (Prescott, 2000).

Transduction is the transfer of resistant genes via a bacterial virus or phage (Prescott, 2000, Schwarz and Chaslus-Dancla, 2001). It is thought to be a relatively unimportant method of resistance transfer because of the specificity of bacteriophages (Prescott, 2000) and the limited amount of space for DNA to be packaged into the phage (Schwarz et al., 2006). Occasionally, resistance plasmids can be accidentally packed up into phage heads during phage assembly and subsequently be able to infect new cells by injecting plasmid DNA into a recipient cell (Schwarz et al., 2006). Neither transformation nor transduction requires a viable donor cell or a link between donor and recipient (Guardabassi and Courvalin, 2006).

Conjugation is the transfer of resistance genes from a resistant organism to a sensitive organism through a protein channel (Bennett, 1995, Prescott, 2000, Schwarz and Chaslus-Dancla, 2001). Gene transfer in conjugation allows the spread of mobile genetic elements such as plasmids, transposons, or integron/gene cassettes (Hall and Collins, 1995, Bennett, 1999, Schwarz and Chaslus-Dancla, 2001). These elements can possess multiple AMR genes and may be responsible for the rapid dissemination of genes among different bacteria (Kruse and Sorun, 1994, Salyers and Cuevas, 1997, Sandvang et al., 1997). Linked clusters of AMR on a single mobile element can also aggregate in such a way that antimicrobials of a different class or even non-antimicrobial substances like heavy metals or disinfectants can select for AMR bacteria (Recchia and Hall, 1997, Salyers et al., 2004). Exchange of resistance genes between pathogens and non-pathogens or between gram-positive and negative bacteria has also been documented (Prescott, 2000, Salyers et al., 2004).

2.4.1.2.1 Mobile genetic elements

As stated above the acquisition of genetic elements such as plasmids, transposons, or integrons/gene cassettes are a critical part of horizontal transfer of AMR. These elements vary considerably from each other in regard to their carriage of resistance, their replication and transmission.

Plasmids are extra-chromosomal circular DNA which can replicate independently, but synchronously with chromosomal DNA (Prescott, 2000, Schwarz et al., 2006). When resistance is transferred as a result of plasmids, a copy of the plasmid is always retained by the parent (Prescott, 2000). Most plasmids carry the gene required for conjugation, but not all do, in these cases plasmids can be mobilized by using the conjugal apparatus of other self-transmissible plasmids within the cell (Prescott, 2000).

Plasmids can code for resistance to between one and ten different antimicrobials (multiple AMR) (Prescott, 2000). Multi-resistant plasmids are often the result of interplasmidic recombination, integration of transposons, or insertion of gene cassettes (Schwarz et al., 2006). All resistance genes on a multi-resistant plasmid are transferred when the plasmid is transferred, whether there is selective pressure for all of the resistance genes on the plasmid or for just one of the resistance genes (Schwarz et al., 2006). Plasmids can act as vectors for transposons and integrons/gene cassettes (Bennett, 1995).

Transposons (jumping genes) are short sequences of DNA that can move from plasmid to plasmid, or from plasmid to chromosome and vice versa (Prescott, 2000). Transposons do not possess replication systems and must be incorporated into chromosomal DNA or plasmids (Schwarz et al., 2006). Unlike plasmids, no copy of the transposon remains within the original cell as the transposon moves between the donor and recipient (Prescott, 2000). All transposons can move and integrate into foreign DNA by nonhomologous recombination, which permits the same transposon to be found in the genome or plasmids of highly unrelated organisms (Prescott, 2000).

Integrons are a mobile element often found on plasmids and are distinct from transposons (Prescott, 2000). They are a site specific recombination system that contains an integrase enzyme, a gene-capture site, and a captured gene or genes (Prescott, 2000). The genes are present as mobile gene cassettes that represent small mobile elements that contain only a single resistance gene and a specific recombination site (Recchia and Hall, 1995, Nandi et al., 2004). The recombination site allows mobility when they are recognized by site-specific integrases, which catalyze integration of the cassettes at specific sites within the integron thereby permitting integrons containing multiple resistance gene cassettes (Prescott, 2000).

Gene expression of an integron is dependent on various factors including promoter strength, gene copy number, the relative distance of the gene cassette from the promoter, and the presence of additional internal promotors (Matinez-Freijo et al., 1998, Martinez-Freijo et al., 1999). Expression is usually mediated via a common promoter

situated upstream (5'-end) of the gene cassettes, rather than through individual promoter copies (Matinez-Freijo et al., 1998). Higher levels of gene expression can be achieved if a second promoter is included adjacent to the first, or if the gene in question is included as multiple copies (Matinez-Freijo et al., 1998). The relative distance between a gene cassette and the promoter plays a significant role regarding expression; proximal genes tend to be expressed more effectively than distal genes (Matinez-Freijo et al., 1998). As a result, distal genes may have very little effect on the susceptibility of the host bacterium to relevant antimicrobials (Matinez-Freijo et al., 1998, Matinez-Freijo et al., 1999). Integron carriage of resistance gene cassettes by the host bacterium was also found to be dependent on the environment that the host organism found itself in, with the loss of integron borne resistance genes in the absence of antimicrobial selective pressure (Rosser and Young, 1999).

2.5. How antimicrobials exert their effect

Antimicrobials by definition are substances that inhibit the growth of or kill micro-organisms with little or no damage to the host. The three main mechanisms of action for antimicrobials to achieve this goal are: inhibition of cell wall synthesis, inhibition of protein synthesis, and inhibition of DNA synthesis. In addition to these three targets antimicrobials may also have an indirect method of action by blocking folic acid synthesis and subsequently inhibiting nucleic acid development.

The cell wall acts as a mechanical means of protection, as a surface for proteins and appendages for cell adhesion, for motility, host infection, and horizontal gene transfer

(Guardabassi and Courvalin, 2006). In gram positive bacteria, the cell wall is thick and composed of multiple layers of cross linked glycan and peptide strands crossed by molecules of teichoic and teichuronic acids. In gram negative bacteria the peptidoglycan wall is thinner and is surrounded by a lipopolysaccharide (Guardabassi and Courvalin, 2006). The lipopolysaccharide layer, in gram negative bacteria, decreases permeability and therefore affects the uptake of certain antimicrobials such as glycopeptides (Guardabassi and Courvalin, 2006).

The main phases of cell wall synthesis are: the cytoplasmic phase, where the peptioglycan layers are formed; the membrane phase, where the muramyl pentapeptide is bound and then transferred to the cell membrane; and the extracytoplasmic phase, where there is crosslinkage (van Heijenoort, 2001). Antimicrobials may exert their effect at any one of these points of cell wall synthesis ultimately leading to the destruction of the cell. The antimicrobials that act primarily on the cell wall include the beta-lactams and the glycopeptides.

Protein synthesis is essential for bacterial survival. It starts with transcription of DNA into mRNA and ends with mRNA translation and translocation (Guardabassi and Courvalin, 2006). Antimicrobials most frequently target translation when the bacterial ribosome reads the mRNA and translates it into amino acid sequences (Guardabassi and Courvalin, 2006). Drugs that act on this step generally bind to specific sites on the ribosome and destroy its functionality (Guardabassi and Courvalin, 2006). Ribosomes are comprised of two subunits, a small 30S subunit and a large 50S subunit

(Guardabassi and Courvalin, 2006). Drugs which target the 30S subunit include aminoglycosides, sepctinomycin, and tetracyclines, while chloramphenicol, pleuromutilins, and oxazolidones target the 50S subunit (Guardabassi and Courvalin, 2006).

Bacterial DNA synthesis permits the replication of the bacterial chromosome during cell division, and RNA synthesis allows gene expression and protein synthesis by transcription of DNA into RNA (Guardabassi and Courvalin, 2006). Quinolones target two enzymes involved in the early stages of this process including topoisomerase II or DNA gyrase and topoisomerase IV, and exert their effect through interaction with the enzyme bound DNA complex (Guardabassi and Courvalin, 2006). Novobiocin also acts on the above two enzymes, but its mechanism of action is through competitive inhibition of ATP by attaching to ATP binding sites; whereas, rifamycins inhibit protein transcription of DNA into mRNA (Guardabassi and Courvalin, 2006).

An additional way that antimicrobials can exert their effect is through the inhibition of nucleic acid synthesis. Sulphonamides and diaminopyramidines (e.g. trimethoprim) have an indirect inhibitory affect on nucleic acid synthesis by blocking various stages of folic acid synthesis (Guardabassi and Courvalin, 2006). Sulfonamides competitively inhibit p-aminobenzoate (PABA) modification into dihydrofolate. Diamionopyramidines competitively inhibit dihydrofolate reductase actively preventing dihydrofolic acid reduction into treahydrofolic acid (Guardabassi and Courvalin, 2006)

2.6. How bacteria fight back against antimicrobials

Resistance to the same antimicrobial can be mediated by several different mechanisms. Additionally, the same resistance gene or mechanism of resistance maybe found in a wide variety of bacteria or limited to certain bacterial species or genera (Schwarz et al., 2006).

The mechanisms that organisms may develop to protect themselves from antimicrobials, and thus become resistant, are often classified into five main categories. These categories include enzymatic inactivation or modification of antimicrobials, impermeability of the bacterial cell wall or membrane, active expulsion of the drug by cell efflux pump, alteration of target receptors, and drug trapping or titration.

1. Enzymatic inactivation or modification of antimicrobials. This is the main mechanism of resistance to beta-lactams, aminoglycosides, and phenicols (Guardabassi and Courvalin, 2006). Drug inactivating enzymes are generally associated with mobile genetic elements. Some of the most clinically important enzymes are the beta-lactamases. These enzymes hydrolyze the beta-lactam ring of penicillins, cephalosporins, and carbapenems preventing them from binding to the active serine site of the penicillin binding proteins (cell wall transpeptidases) and impede cell wall synthesis (Guardabassi and Courvalin, 2006). The other clinically important enzymes are the aminoglycoside modifiying enzymes that catalyze the transfer of an acetyl group (*N*-acetyltransferases), a phosphoryl group (O-phosphotransferases), or a nucleotide

(O-nucleotidyltransferases) to the amino or hydroxyl group of the aminoglycoside molecule. The end result is a chemically modified drug that has poor binding to the ribosomes and is subsequently not taken up by the cell (Wright, 1999).

2. Impermeability of the bacterial cell wall or membrane. Hydrophyllic drugs enter gram negative bacterial cell wall through porins, while hydrophobic drugs enter through the phospholipid layer (Guardabassi and Courvalin, 2006). Any change in porins can confer resistance (Guardabassi and Courvalin, 2006). Lack of aminoglycoside activity in anaerobes is a result of reduced drug uptake.
3. Active expulsion of the drug by cell efflux pump. Efflux pumps are proteins that reduce the concentration of the drug in the cytoplasm thereby limiting access of the drug to its target (Guardabassi and Courvalin, 2006). There are two major types of antimicrobial efflux pumps (Guardabassi and Courvalin, 2006). The first type of efflux pump acts on specific drugs. Drug pumps are an important mechanism for tetracycline resistance especially in gram negative bacteria, but can also confer resistance to phenicols (Guardabassi and Courvalin, 2006). Specific drug pumps are associated with mobile genetic elements (Butaye et al., 2003). The second type of efflux pump creates multiple drug resistance. This pump is frequently encoded by the chromosome and is divided into ATP binding cassette transporters or secondary drug transporters depending on their source of energy (Putman et al., 2000). The secondary drug transporters are the pumps that account for most of the resistance to multiple antimicrobial agents.

4. Alteration of target receptors. Resistance by alteration or protection of drug receptors has been reported for tetracycline and or quinolones (Guardabassi and Courvalin, 2006).
5. Drug trapping or titration can be accomplished by several venues and the consequence is reduced free drug at the target site (titration). For example, the chromosomal mutations responsible for the overproduction of PABA, the target of sulphonamides and diamionpyrimidines (dihydrofolate reductase), have been reported in several bacteria (Guardabassi and Courvalin, 2006).

2.6.1. Primary mechanisms of antimicrobial resistance development of six antimicrobials important in cow-calf herds

Schwarz et al. (2006) provide a good over view of the mechanisms and spread of bacterial resistance to nine classes of antimicrobial agents that play a major role in veterinary medicine, as well as for glycopeptides and streptogramins which are important in human medicine. Brief descriptions of the primary mechanisms for AMR development of six antimicrobial classes commonly used in cow-calf operations are provided here.

2.6.1.1. Beta-lactams

Resistance to beta-lactam antimicrobials is mainly due to inactivation by beta lactamases (Livermore, 1995) and decreased ability to bind to penicillin-binding proteins (Georgeopapadakou, 1993). However, beta-lactam resistance may also be a

result of decreased uptake of the drug due to permeability barriers or increased efflux via multidrug transporters (Paulson et al., 1996, Quintiliani et al., 1999). The inactivation of beta-lactams is primarily due to the cleavage of the amino bond in the beta-lactam ring by a beta lactamase enzyme (Bush et al., 1995, Livermore, 1995, Bush, 2001, Wiegand, 2003). Genes encoding beta-lactamases are located on either plasmids or the bacterial chromosome (Aarts et al., 2006). Examples of specific gene variants for the beta-lactamase family in gram negative bacteria include ampC, tem, shv, oxa and ctx-M (Aarts et al., 2006). Extended spectrum beta-lactamases that play an important role in human medicine have also been described (Bradford, 2001), as have the AMR genes for methicillin resistant Staphylococcus aures (Aarts et al., 2006).

2.6.1.2. Tetracyclines

Tetracycline resistance is almost always a result of the uptake of new genes (Chopra and Roberts, 2001). There are 23 efflux genes (which code for energy dependent efflux of tetracyclines), 11 ribosomal protection genes (which code for protein that protects bacterial ribosomes), 3 genes that code for enzymes that modify and inactivate tetracycline, and 1 gene that has an unknown mechanism (Schwarz et al., 2006). Currently only the first two mechanisms are important in bacteria of veterinary importance.

The efflux resistance genes *tet*A, *tet*B, *tet*C, *tet*D and *tet*H are most wide spread in gram negative bacteria and are located on transposons (Allmeier et al., 1992, Chalmers et al., 2000, Lawley et al., 2000) and plasmids (Schwarz et al., 2006). The *tet*B gene

confers resistance to both tetracycline and minocylcine, but not to the new glycyclines, while the other efflux proteins confer resistance only to tetracycline (Chalmers et al., 2000, Chopra and Roberts, 2001). Resistance to minocycline and glycyclines are relevant as they are newer drugs that play a role in human medicine. The methodologies utilized to identify these different *tet* resistance genes have been described elsewhere (Frech and Schwarz, 2000, Kehrenberg et al., 2001, Aminov et al., 2001, Ng et al., 2001, Guerra et al., 2004, van Hoek et al., 2005).

Ribosomal protection genes are a second important way for tetracycline resistance development. They are of gram positive origin but can also be found in gram negative genera (Schwarz et al., 2006). An example of a ribosomal protection gene is the *tet*M gene which has a wide range of hosts and is located on a conjugative transposon (Flannagan et al., 1994, Chopra and Roberts, 2001, , Salyers et al., 1995).

Other less well described mechanisms of tetracycline resistance include enzymatic inactivation, 16S rRNA mutation, other mutations, and multidrug transporters (Schwarz et al, 2006).

2.6.1.3. Quinolones and Fluorquinolones

Quinolones and fluoroquinolones are potent inhibitors of bacterial DNA replication (Schwarz et al., 2006). The two major mechanisms of resistance development to fluorquinolone antimicrobials are point mutations and decreased intracellular accumulation (Schwarz et al., 2006). Several recent reviews deal with the molecular

basis and epidemiology of quinolone resistance in *E. coli* and *Salmonella* spp. of animal origin (Drlica and Zhao, 1997, Everett and Piddock, 1998, Hooper, 1999, Bager and Helmuth, 2001, Cloeckaert and Chaslus-Dancla, 2001, Webber and Piddock, 2001, Ruiz, 2003).

Briefly, point mutations in the target genes *gyr*A and *gyr*B coding for DNA gyrase and or for *par*C and *par*E coding for DNA topoismerase IV are frequent in quinolone and fluoroquinolone resistance (Schwarz et al., 2006). Detection of these point mutations in the region of the *gyr*A, *gyr*B, or *par*C and *par*E genes can be accomplished through PCR (Aarts et al., 2006) while microarrays have been used to assess mutlidrug efflux systems. Resistance genes associated with multidrug efflux pumps vary depending on the organism involved (Schwarz et al., 2006) and they may lead to high levels of resistance to quinolones and other antimicrobials where multidrug efflux pumps and decreased membrane permeability are involved (Lee et al., 2000). Quinolone and fluorquinolone resistance can also result from interaction between different resistance mechanisms, decreased drug uptake and DNA gyrase protection (Schwarz et al., 2006)

2.6.1.4. Aminoglycosides and Aminocyclitols

The main mechanism for aminoglycoside resistance is enzymatic inactivation (Shaw et al., 1993, Mingeot-Leclercq et al., 1999), but reduced uptake and chromosomal mutations conferring high levels of resistance to streptomycin have also been described (Quintiliani et al., 1999). Aminoglycoside resistance is mediated by more that 50

aminoglycoside modifying enzymes that are classified as either aminoglycoside acetyltransferases (*aac*), aminoglycoside andenyltransferases (*aad* or *ant*), and aminoglycoside phosphotransferases (*aph*) (Shaw et al., 1993, Mingeot-Leclercq et al., 1999, Aarts et al., 2006). Most *aac,* ant and *aph* genes are located on mobile genetic elements such as plasmids, transposons, or gene cassettes (Shaw et al., 1993, Recchia and Hall, 1995, Davies and Wright, 1997, Mingeot-Leclercq et al., 1999, Wright, 1999, Sandvang and Aarestrup, 2000,). The modifications of aminoglycosides and aminocyclitols by inactivating enzymes have been described in detail in various reviews (Shaw et al., 1993, Davies and Wright, 1997).

2.6.1.5. Chloramphenicol and Florfenicol

Both enzymatic and non-enzymatic chloramphenicol and florfenicol resistance genes have been described (Aarts et al., 2006), but enzymatic inactivation is the predominant method (Shaw, 1983, Murray and Shaw, 1997, Schwarz et al., 2004) of resistance development. Enzymatic resistance genes are primarily encoding acetyltranfereases and are the *cat* genes (Aarts et al., 2006). Non-enzymatic gene coding of chloramphenicol and florfenicol include the *cml* genes on transposon TN1696 and the *flo*R gene (Aarts et al., 2006). Efflux systems conferring resistance to chloramphenicol alone or in combination with florfenicol (Schwarz et al., 2004), permeability barriers, and multidrug transporters (Paulsen et al., 1996, Schwarz et al., 2004) as well as other minor mechanisms of resistance have also been identified for this class of antimicrobials (Schwarz et al., 2006). Details on different genes and mechanisms for phenicol resistance are available (Schwarz et al., 2004)

2.6.1.6. Sulphonamides and Trimethoprim

Sulphonamides and trimethoprim are competitive inhibitors of different enzymatic steps in folate metabolism (Schwarz et al., 2006). Sulphonamide resistance can result from chromosomal mutations in the dihydropteroate synthase (*fol*P) gene or by acquisition of resistant dihydropteroate synthase genes (*sul* genes) (Aarts et al., 2006, Schwarz et al., 2006). Three *sul* genes have been described in gram negative bacteria (Swedberg and Skold, 1980, Radstrom and Swedberg, 1988, Aarts et al., 2006). The *sul*I gene is associated with class 1 integrons and, therefore, is often linked to other genes. It is spread in gram negative species as part of transposons or as conjugative plasmids (Sundstrom et al., 1988). The *sul*II gene often occurs with streptomycin resistance genes *str*A and *str*B on conjugative or nonconjugative plasmids (Radstrom and Swedberg, 1988, Kehrenberg and Schwarz, 2004), while the *sul*III gene can be found on conjugative plasmids (Perreten and Boerlin, 2003)

Trimethoprim resistance is primarily mediated by acquisition of *dfr* gene encoding resistant dihydrofolate reductase (Aarts et al., 2006, Schwarz et al., 2006). Transferable trimethoprim resistance has been identified in a variety of gram negative bacteria and several of these genes are part of plasmids, transposons, or gene cassettes (Recchia and Hall, 1995, Skold, 2001, Ito et al., 2004). Other potential mechanisms of trimethoprim resistance for some bacteria include permeability barriers and efflux pumps (Kohler et al., 1996, Huovinen, 2001) and *dhfr* and folate auxotrophy (Quintiliani et al., 1999). Mutations in chromosomal genes have also been observed (Huovinen, 2001).

2.6.2. Co-resistance and cross resistance

The development of AMR is a complex process and the speed with which it develops depends on the bacteria involved, the selective pressure, and the availability and transferability of resistance genes (Schwarz et al., 2006). Recent studies have shown that the majority of multiple resistant phenotypes are obtained by the acquisition of external genes that may provide resistance to an entire class of antimicrobials (White and McDermott, 2002). When there is the selection of multiple AMR genes when one gene is selected this is called co-selection.

Co-resistance is the coexistence of several different mechanisms including genes or mutations which allow bacteria of the same strain to be resistant to a variety of related or non-related substances simultaneously (Weldhagen, 2004, Guardabassi and Courvalin, 2006). Beta-lactamase genes that are situated on the class 1 integron can be used to provide four examples of co-resistance. Co-resistance can occur when beta-lactamase genes on class 1 integrons are found with genes to quaternary ammonium compounds and sulphonamides (*sul*I) that classically occur at the distal 3'-end (Poirel et al., 2000, Poirel et al. 2001, Dubois et al., 2002). A second example is the common co-existence of aminoglycoside encoded gene cassettes with beta-lacatamase gene cassettes on integron structures (Weldhagen, 2004). A third example is *cat*-type and *clm*-type gene cassettes co-existing on class 1 integrons with class A and class B beta-lacatamase genes (Weldhagen, 2004). Finally, a fourth example is the occurrence of *sul*-

gene types and the *dhfr*I/*dhfr*-gene types occurring together on class 1 integrons in conjunction with class A beta-lactamase *bla* gene cassettes (Weldhagen, 2004).

Cross resistance is a single biochemical mechanism in which resistance to one drug is associated with resistance to another drug of the same or different class (Guardabassi and Courvalin, 2006). For example, despite differences in their chemical structure cross resistance to macrolides, lincosamides, and beta-streptogramins can result from the methylation of a single adenine residue in 50S rRNA (Guardabassi and Courvalin, 2006). When the biochemical mechanism is drug efflux then cross-resistance to several drugs may be observed (Courvalin and Tri-Cuot, 2001). Cross-resistance is common among macrolides and fluoroquinolones (Prescott, 2000).

2.7. *Escherichia coli (E. coli)* as an indicator organism

Many AMR studies have focused on organisms that are pathogenic to humans including: *Salmonella* spp. (Wray et al., 1991), *Campylobacter* spp. (Gaunt and Piddock, 1996), or *Escherichia coli* O157 (Meng et al., 1998). However, transmissible genetic elements encoding AMR can also be maintained in commensal bacteria (Shaw and Cabelli, 1980, Falagas and Siakavelllas, 2000). Resistance gene transmission from normally nonpathogenic species to more virulent organisms within the animal or human intestinal tract may be an important mechanism for acquiring clinically significant antimicrobial resistant organisms (Winokur et al., 2001). *E. coli* have developed a number of elaborate methods for acquiring and disseminating genetic determinants and,

therefore, may serve as an important reservoir for transmissible resistance (Neidhardt, 1996).

Commensal *E. coli* have been included in various surveillance programs and research projects as indicators of both selection pressure and the reservoir of resistance genes. The rationale for using *E. coli* includes: it is commonly found in avian and mammalian species and, therefore, a good benchmark for comparison between species and ecological niches; it is easy to grow; and there is the opportunity for the spread of transferable resistance. There is evidence that resistance transfer occurs *in vivo* between *E. coli* and other *E. coli*, other *Enterobacteriacea*, and other types of bacteria (Winokur et al., 2000, Winkokur et al., 2001). *E. coli* from fecal specimens also make good indicator organisms since they carry more resistance markers than any other *Enterobacteriaceae* species in the gut (Osterblad et al., 2000). Investigating AMR in generic *E. coli* from domestic species is, therefore, a practical way to improve the understanding of AMR ecology and the potential role of commensal microbiota of mammals as a reservoir for AMR.

2.8. Shiga toxin producing *E. coli* and AMR

Shiga toxin or verotoxin producing *E. coli* (STEC/VTEC) are the most important recently emerged group of foodborne pathogens (Remis et al., 1984, Karmali, 1989, Beutin et al., 1998, Paton and Paton, 1998, Beutin et al., 2002, Blanco et al., 2004, Mora et al., 2004,). Major STEC associated outbreaks have been experienced in Canada, Japan, the United Kingdom, and the United States (Karmali, M., 1989, Beutin

et al., 1998, Paton and Paton, 1998, Willshaw, et al., 2001, Beutin et al., 2002). In humans, these infections are associated with gastroenteritis that may be complicated by hemorrhagic colitis (HC) or hemolytic-uremic syndrome (HUS), which is a major cause of renal failure in children (Mora et al., 2004).

STEC's produce either one or two cytotoxins called Shiga toxins (*stx*1 and *stx*2) or verotoxins (*vt*1 and *vt*2) (Paton and Paton, 1998). Intimin is another virulence factor responsible for intimate attachment of STEC. It is encoded by chromosomal gene *eae* which is part of a large cluster of virulence genes on a pathogenicity island termed the locus for enterocyte effacement (LEE) (Kaper et al., 1998). STEC's carrying the *eae* gene have been closely associated with HC and HUS (Karmali, 1989).

Generally antimicrobials are not recommended for therapy of STEC infections because antimicrobials can lyse cell walls therefore releasing the toxins (Waterspiel et al., 1992, Wong et al., 2000). Additionally, antimicrobials are usually avoided because they can also cause increased expression of the toxins *in vivo* (Zhang et al., 2000). Despite the general practice of not using antimicrobials to treat STEC infections, there have been recent reports suggesting that AMR of STEC is on the rise (Gonzalez et al., 1989, Farina et al., 1996, Meng et al., 1998, Galland et al., 2001, Willshaw et al., 2001, Schroeder et al., 2002,).

Virulence genes are either located in chromosomal gene clusters (pathogenicity islands) or harbored in mobile accessory genetic elements such as plasmids and phages

(Groismann, 1996, Finlay and Falkow, 1997, Hacker et al., 1997). Resistance genes are also often associated with mobile DNA such as plasmids, transposons, and integrons (Jacoby, 1994, Tenover and Rasheed, 1998). Since AMR and virulence genes are carried in a similar fashion, it is possible that AMR and virulence genes could be linked and then co-selected (Martinez and Baquero, 2002). Therefore, reported increases in antimicrobial resistant STECs are of concern because AMU could potentially enhance the selection for bacteria carrying virulence genes. Antimicrobial use could ultimately accelerate the spread of virulence genes within bacterial populations and enhance the emergence of new pathogens or of pathogens with increased virulence potential (Boerlin et al., 2005). Finally, resistance genes may also be stabilized and fixed in pathogen populations by their linkage to virulence genes (Boerlin et al., 2005).

Domestic ruminants especially cattle, sheep, and goats have been implicated as the primary reservoirs for STEC (Blanco et al., 1997, 2001, 2003, 2004). AMR from livestock and farms pose a potential risk to public health through direct contact with livestock or production environments, but also through AMR food-borne pathogens (van den Bogaard and Stobberingh, 2000, White et al., 2001). Non-pathogenic *E. coli* are also considered a problem because they can provide a pool of transferable resistance genes (Schmieger and Schicklmaier, 1999, Winokur et al., 2001).

The current literature contains several articles describing AMR in STEC's from a variety of populations (Blanco, 1989, Galland et al., 2001, Zhao et al., 2001, Gonzalez and Maidhof et al., 2002, Schroeder et al., 2002, Betteleheim et al., 2003, Mora et al.,

2005). However, the current literature describing AMR in STEC does not always contain directly comparable information on the nature and extent of resistance in non-STEC populations. The presence of AMR within a STEC positive isolate in the absence of additional information does not indicate whether or not STEC are more or less likely to be resistant to antimicrobials than non-STEC organisms, or conversely whether organisms that are antimicrobial resistant are more or less likely to contain virulence genes than organisms that are sensitive. This particular area needs more research to fully understand what is happening in STEC and non-STEC populations.

2.9. Antimicrobial use: General considerations

Different AMU regimen select for various resistance genes (Blake et al., 2003), and, therefore, use patterns of antimicrobial agents are expected to have some impact on the distribution of antimicrobial resistant phenotypes (McGowan and Gerding, 1996, Gaynes and Monnet, 1997, Aarestrup, 1999). Among the ramifications associated with such resistance gene selection are the degree of resistance conferred and the carriage of linked resistance determinants (Blake et al., 2003). Minimal antimicrobial exposure is necessary to select for continued persistence of resistance genes within enteric microflora (Blake et al., 2003). Persistence of AMR in bacteria is related to the persistence of antimicrobials. Therefore, short-term therapeutic treatment with antimicrobials generally do not produce bacteria that persist in the intestine, whereas prolonged AMU is more likely to be associated with persistence of resistant organisms after the drug is no longer administered (Prescott, 2000). As a result of the effect of

AMU on AMR, it is important to consider how AMR may be affected in a variety of production systems and livestock species with different AMU patterns and intensities.

2.9.1. Reason for antimicrobial use in livestock

Antimicrobial use in livestock production is necessary for the health and welfare of the animals. Method of administration and the volume of antimicrobial used will vary depending on the species of livestock, stage of production, and risk of disease. There are three primary reasons for AMU in food-producing animals: treatment of diseased animals, prevention and control of disease, and growth promotion.

Prevention and control can be further divided into metaphylactic or prophylactic applications. Metaphylaxis is a disease control measure involving the mass medication of a group of animals to prevent the spread of disease when only a few individuals have been identified as infected. Prophylaxis is a preventative treatment of an animal or group of animals at a time when it may be more susceptible to infection. An example of prophylactic use of antimicrobials includes the treatment of dairy cows at the end of lactation. Antimicrobials are given at critical points in production to help prevent the development of disease. Prophylactic treatment may involve the entire group of animals or may be targeted towards specific high risk individuals depending on the animal species, the production system, and the disease condition.

While there may be concern about the impact of metaphylactic and prophylactic treatment of groups of animals on the development of AMR, the bigger concern is the

use of antimicrobials for growth promotion. Growth promotion generally involves the use of antimicrobials licensed for this purpose. Generally antimicrobials used for growth promotion are provided at a sub-therapeutic dose (dose lower than those approved for therapeutic purposes) and are fed for a longer duration than antimicrobials used for prevention and control. It is the lower dose and the long duration of feeding of these antimicrobials which often causes concern about the development of AMR.

Intensive livestock operations, such as feedlots, swine, or poultry operations, are often required to use the tools of prophylactic, metaphylactic, and growth promotant AMU in order to prevent disease and death, to ensure animal welfare, and for economic benefit. Cow-calf herds are generally managed more extensively and, therefore, these operations would be less likely to use antimicrobials in this way. In cow-calf herds the use of antimicrobials to prevent or control disease may be necessary in disease outbreak situations or in facilities with less than optimal management practices.

2.9.2. Antimicrobial use and antimicrobial resistance

The Canadian Veterinary Medical Association (CVMA) has taken a pro-active stance on AMR (CVMA, 2005) and makes several general recommendations. The first is that veterinarians, animal owners, and animal caretakers all share a responsibility for minimizing the use of antimicrobial drugs to conserve drug efficacy (CVMA, 2005). Veterinarians have a responsibility to educate staff, clients, and other animal handlers on the prudent use of antimicrobials and for ensuring such training occurs (CVMA, 2005). The role of the veterinarian is to ensure that all users are aware of the appropriate

administration, handling, storage, disposal, and record keeping for antimicrobials (CVMA, 2005).

The CVMA (2005) also recommends that veterinarians should continually update their knowledge of disease prevention, therapeutics, and of issues such as drug resistance trends to ensure the prudent use of antimicrobials. Implementation of preventative measures such as vaccination, biosecurity measures, good hygiene practices, and improved management may help prevent disease and, therefore, reduce the use of antimicrobials. Additionally, if the veterinarian understands resistance patterns that are emerging on a farm, they will be better able to make recommendations regarding antimicrobial treatment.

Finally, the CVMA (2005) recommends that all antimicrobials even those not purchased directly through or on prescription from a veterinarian, should be used within the confines of a valid veterinarian-client-patient relationship. This should help ensure appropriate AMU because of the veterinarian's understanding of farm management and disease status. With this knowledge, antimicrobial treatments can be designed to maximize therapeutic efficacy and minimize bacterial resistance.

Both the structures and biochemical pathways within bacteria that determine antimicrobial availability within the microbe and the pharmacodynamic and pharmacokinetic properties of antimicrobials are complex (Aliabadi and Lees, 2000). Rational dosing of antimicrobials depends upon knowledge of physiology, anatomy,

pathology, and disease condition (Lees and Aliabadi, 2002). Resistance can result from both the selection of an inappropriate antimicrobial and from failure to optimize the dose level. For example, the dose interval is important for concentration-dependent antimicrobials and the duration of treatment is critical for time-dependent antimicrobials (Aliabadi and Lees, 2000). Pharmacokinetic variation may result from the animals' disease status, age, and weight, or from non-biological factors such as route of administration, formulation, and drug interactions (Aliabadi and Lees, 2000). Through the optimization of the dosage schedule, the beneficial effects of treatment are maximized while the potential adverse effects are minimized (Aliabadi and Lees, 2000). While implementing the appropriate use of antimicrobials may limit AMR development, is it also important to recognize that even appropriate use can put selection pressure on the bacterial population and contribute to AMR.

Producer access to over the counter (OTC) antimicrobials often results in AMU with little or no veterinary consultation (McEwen and Fedorka-Cray, 2002). These OTC drugs are made available to producers for purely practical reasons such as lack of access to a veterinarian (McEwen and Fedorka-Cray, 2002). This practice can result in inappropriate antimicrobial choices, dosing and treatment frequency and, therefore, may be a factor in AMR development.

The continued availability of antimicrobials in veterinary medicine depends upon the profession's ability to use these products wisely and find the balance between maximizing animal welfare and conserving antimicrobial efficacy (CVMA, 2005). By

increasing awareness about AMR and implementing prudent use recommendations veterinarians can promote long term efficacy and continued availability of antimicrobials.

Probably one of the most important methods of avoiding resistance is ensuring that antimicrobials are selected and used appropriately. They should not be used as a substitute for poor hygiene or poor disease control. Ensuring that all individuals using antimicrobial are well informed about when, where, and how antimicrobials should be used is an important step in avoiding further resistance development. Emphasizing the importance of preventative health programs along with good management and hygiene practices on the farm will reduce the potential for disease and the need for AMU. Additionally, monitoring programs can also help illustrate changing resistance patterns over time to alert us to new or emerging resistance patterns.

2.9.3. Challenges of antimicrobial use data collection

Antimicrobial use data are difficult to collect and report for several reasons. National, regional, or even farm level data are scarce. On a national level, Denmark, Sweden, and Finland are currently the only countries where pharmaceutical companies have a legal obligation to supply data on antimicrobial sales (Schwarz and Chaslus-Dancla, 2001). At the national level, AMU is reported as kilograms or tonnes of active ingredient sold. While these data provide the volume of antimicrobial used it does not allow for drug potentcy, assessment of how the antimicrobial was used and whether this use may affect AMR. Data on the species it was delivered to, the number of animals

exposed, the dose received, and the method of delivery are generally unavailable. End-user data are, therefore, required in order to gain a better appreciation of how and why antimicrobials are being used in livestock production, but even end-user AMU data has serious limitations.

Complete and accurate farm-level AMU records are difficult to obtain. Capture of use information can be expensive for the researcher and burdensome for the producer to accommodate especially during times of additional demands with limited resources. Under reporting is potentially a problem since producers are busy with day-to-day operations on the farm and, therefore, record keeping may be relatively low on the priority list. Subsequently treatment records may be forgotten or incomplete. Dunlop et al. (1998) reported a 35% under-reporting rate for AMU recorded by swine producers as compared to inventory and disappearance data collected by the researcher. A preliminary report of AMU in the Ontario beef industry, by Bair and McEwen, (2001) estimated average under reporting of AMU on farm was 40%. This estimate was based on treatment diaries and accounting of drug disappearance in both feedlots and cow-calf herds (Bair and McEwen, 2001). For cow-calf herds specifically, under reporting ranged from 1-86% with a mean of 24% (Bair and McEwen, 2001). Several reasons for under-reporting include: misunderstandings between researchers and producers, and lack of time during periods of increased work load such as in disease outbreak situations (Singer et al. 2006).

In addition to the challenges of collecting AMU data, there is no widely accepted method for quantifying AMU (Singer et al., 2006). Use can be reported in many ways including, but not limited to, total volume of drug in kilograms, defined daily doses (DDD) (Jensen et al. 2004), animal daily doses (ADD) (Jensen et al., 2004), or as animal-units per treatment days (Dunlop et al, 1998). While each of the above methods try to capture the true exposure of an animal to a antimicrobial, they all are limited. Debate still surrounds the best approach to reporting AMU information. Jensen et al. (2004) provide a good overview and highlight the major potential methods for reporting AMU and the associated limitations.

Although there are several issues associated with AMU data capture and reporting there is international interest in developing surveillance systems for AMR and AMU that potentially includes farm-level or aggregate-level AMU data (Rosdahl and Pederson, 1998, Nicholls et al., 2001 and WHO 2001). In order to achieve a meaningful way to collect and report AMU data, international collaborative efforts are being made to overcome the issues surrounding AMU collection. For end-user compiled AMU data to be useful in surveillance systems, the following pieces of information need to be collected: total amount of antimicrobial used, indication for treatment, route of administration, dose and duration (Singer et al., 2006).

2.10. Antimicrobial use and antimicrobial resistance in cow-calf herds

Little information is available on AMU and AMR in cow-calf herds. In papers describing AMU (Bair and McEwen, 2001, Powell and Powell, 2001, Busani et al.,

2004, Sayah et al., 2005) or AMR in healthy cattle it is often not differentiated as to what was the age and type of cattle sampled (Mercer et al., 1971, Schroeder et al., 2002, Kijima-Tanaka et al., 2003, Sayah et al., 2005, Lim et al., 2007). If the literature pertains to beef cattle specifically the samples collected are often at the abattoir rather than on farm (Van Donkersgoed et al., 2003, CIPARS, 2006, Aslam and Service, 2006, Rigobelo et al., 2006). While abattoir samples provide some insight into on farm prevalence they may not completely reflect AMR patterns on farm. Also, the ability to link on farm AMU with AMR is critical in order to be able to identify risk factors and the potential impact of certain farm management practices.

The following review illustrates the limited amount of data that are available on cow-calf AMU and AMR and the gaps that need to be filled. Additionally it highlights the effects of management practices, AMU, animal age, and potentially other host specific factors as they relate to AMR.

2.10.1. Antimicrobial use

Antimicrobial use is generally accepted to be an important factor for the selection of AMR bacteria (Aarestrup, 1999, van den Bogaard and Stobberingh, 2000, McEwen and Fedorka-Cray, 2002). Selective pressure for AMR can be affected by treatment formulation, dose, interval and duration (Catry et al., 2003). Since there can be diverse AMU practices within the livestock industry, describing AMR in one livestock class, species, or management system can not be considered to be representative of another.

Many studies have been initiated to investigate a potential link between AMU and subsequent AMR in animals and the development of resistance in people (Hummel et al., 1986, Endtz et al, 1991, Johnson et al., 1995, Bager et al., 1997, Aarestrup, 1999, Winokur et al., 2001, Swartz, 2002). Additionally, studies of commensal and pathogenic resistant bacteria have been conducted in a variety of livestock species in order to more fully understand the type and level of resistance that is present in livestock (Dargatz et al., 2003, Fiztgerald et al., 2003, Lanz et al., 2003, Bywater et al., 2004, Khachatryan et al., 2004, Rajic et al., 2004, Hershberger et al., 2005). Many of these studies have focused on intensively reared livestock populations, such as feedlots, swine, and poultry operations, that may incorporate antimicrobials in feed for prophylactic, metaphylactic, or therapeutic reasons (McEwen and Fedorka-Cray, 2002).

In addition to in feed AMU, the dairy or feedlot industry may also use antimicrobials prophylactically in a substantial proportion of individual animals. Between 75-90% of all dairy cattle receive prophylactic antimicrobials to prevent mastitis (Sishco et al., 1993, USDA, 2003). Depending upon the size of the feedlot, the type of cattle placed and the bovine respiratory disease risk designation, anywhere between 16-19% of feedlot cattle in the United States (USDA, 1999) and 20-50% of feedlot animals in Canada receive prophylactic injectable antimicrobials on arrival for the control of bovine respiratory disease (Radostits OM, 2001, personal communication with Dr. Calvin Booker, FHMS, February 22, 2007).

In contrast to these more intensive livestock management systems, cattle in cow-calf herds in western Canada are primarily raised extensively and are, therefore, subjected to different management practices and antimicrobial exposures than livestock species that are raised more intensively. The typical production cycle for cows calving in the winter-spring months of the year involves a period of confinement to pens or small pastures that enable producers to readily observe the cattle prior to and during the calving season. The duration and intensity of this confinement varies between farms and management systems. In many herds, cows that are due to calve are kept separate from cows that have already calved. Upon completion of the calving season and depending on grass accessibility, cow-calf pairs are then turned out of these more confined areas onto larger pastures. Cattle may be kept on these larger pastures into the fall and earlier winter depending on availability of feed and weather conditions. Calves are usually weaned in the fall, and at that time they may be sold or kept as replacement animals (Mathison, 1993).

Because of management and environmental conditions, in-feed use of antimicrobials and routine injectable AMU in cow-calf herds is assumed to be less frequent than in other species. However, there are very limited data available to understand the selective pressures experienced in cow-calf herds. Four papers, two published and two un-published, containing data on AMU in cattle were identified. In some cases it is not always clear whether the data provided pertains specifically to cow-calf herds, to beef cattle, or to cattle in general. This makes it very difficult to draw any conclusions about AMU practices within cow-calf herds.

A Michigan study of AMU in cattle collected information for 60 days prior to the administration of the questionnaire (Sayah et al., 2005). This study indicated that, during this time, no beef cattle were treated with any of the following drugs: streptomycin, trimethoprim-sulphamethoxazole, tetracycline, ampicillin, cloxacillin, or bacitracin (Sayah et al., 2005). The following drugs were used on 89 beef cattle reported as treated: enrofloxacin (2.5%), sulphamethazine (55.7%), chlortetracycline (55.7%), oxytetracycline (16.5%), penicillin (2.5%), ceftiofur (1.3%), and tilmicosin (27.9%)(Sayah et al., 2005). Dairy cattle on the other hand were treated differently (n=131) (Sayah et al., 2005) than the beef cattle. Streptomycin was used on 32.8%, sulphamethazine in 20.6%, trimethoprim-sulphamethoxazole in 1%, tetracycline, chlortetracycline and oxytetracycline in 12.7%, 21.4%, and 16.8% of the dairy cattle respectively (Sayah et al., 2005). Penicillin, ampicillin, cloxacillin, and ceftiofur were also used in 60.8%, 3.1%, 2.3%, and 19.9% of dairy cattle (Sayah et al., 2005).

Bair and McEwen (2001) looked at AMU in the Ontario beef industry. This study included 16 feedlots and 13 cow-calf farms. The preliminary report indicated that oxytetracycline and tilmicosin were used prophylactically on 6/16 feedlots, whereas oxtetracycline or penicillin were used prophylactically on 3/13 cow-calf farms (Bair and McEwen, 2001). All feedlots used ionophore medication in the feed (Bair and McEwen, 2001). Nine of 13 cow-calf herds used ionophores in feed (Bair and McEwen, 2001). No cow-calf herds used any other in-feed antimicrobials (Bair and McEwen, 2001). One feedlot and 1 cow-calf herd used water medication (Bair and McEwen, 2001). A sulpha

based drug was used in the feedlot and chlortetracycline was used in the cow-calf herd (Bair and McEwen, 2001). The most commonly used injectable drugs were: penicillin (45% of herds), florfenicol (35%), ceftiofur (17%), oxytetracycline (22%), tilmicosin (80%), and sulbactam-ampicillin (11%).

A survey of Ontario beef producers' attitudes about AMU was issued in 1999 (Powell and Powell, 2001). A 4% response rate was achieved (Powell and Powell, 2001). This represented 8% of the market steers, 7.5% of the market heifers, and 4.0% of the beef cows in Ontario (Powell and Powell, 2001). Of the 587 valid responses, 341 came from cow-calf herds and 106 were from feedlots (Powell and Powell, 2001). The average cow-calf herd size was 40 animals, where half of the farmers reported 20 cows or less (Powell and Powell, 2001). The most commonly reported illness was diarrhea followed by respiratory disease (Powell and Powell, 2001). Approximately 1/3 of the farms reported treating less than 5% of their animals (Powell and Powell, 2001). Antimicrobial cost was determined to be an important factor in determining whether an antimicrobial was used or not. Most (94.4%) of respondents agreed that it is important to reduce AMU (Powell and Powell, 2001). The majority (78.0%) had concerns about negative media coverage and felt that his would cause them to re-evaluate their use practices (Powell and Powell, 2001). The injectable products used included: oxtetracycline (50.0%), penicillin (48.5%) tilmicosin (27.2%), trimethoprim-sulphadoxine (23.0%), and florfenicol (14.6%) (Powell and Powell, 2001). Off label use of enrofloxacin was also reported on some farms (<1.0% of respondents) (Powell and Powell, 2001). Over half of the producers in this survey also did not know the

difference between an antimicrobial and other injectable products (Powell and Powell, 2001). Prophylactic use was reported by 65 respondents, and 19 respondents reported treating the entire herd when only some animals were sick (Powell and Powell, 2001). A strong relationship was seen between treating less than 10% of the animals and having a regular veterinarian (Powell and Powell, 2001).

Busani et al., (2004) did a similar survey to Powell and Powell (2001), but targeted beef and dairy cattle veterinarians rather than producers. This telephone survey included 106 veterinarians; 62% treated only diary cattle, 10% treated only beef and 28% treated both (Busani et al., 2004). When treating mastitis, enteritis in calves, and when treating respiratory disease, laboratory analysis was requested always or frequently by 67.0%, 37.0% and 17.0% of the veterinarians respectively. Prophylactic AMU for calf enteritis was used by 20% of veterinarians often or sometimes. For respiratory disease, 28% of veterinarians used prophylactic antimicrobials often or sometimes, and for mastitis, 62% of the veterinarians reported using antimicrobials prophylactically always or often. Veterinarians reported using fluoroquionolones, phenicols, or third generation cephalosporins as the drugs of first choice for scours (54%), respiratory disease (12%), and mastitis (6%). Most veterinarians were aware of the problem of AMR (94%).

2.10.2. Reason for treatment in cow-calf herds

To date there is also very little information on reason for treatment and AMR in the beef industry particularly in cow-calf herds, and there is no information available on western Canadian cow-calf herds. The National Animal Health Monitoring System

(USDA) is the only available source that provides some insight into the reason for treatment in cow-calf herds. USDA (1997) reported a relatively low occurrence of disease and treatment in breeding females of cow calf herds. Pinkeye and interdigital necrobacillosis were listed as the two primary disease conditions reported among breeding females; whereas, diarrhea, followed by pneumonia, were the two most commonly reported illness in beef calves (USDA, 1997).

2.10.3. Antimicrobial resistance

The most common resistances detected in fecal *E. coli* isolates in a variety of species has been to tetracycline, sulphamethoxazole, and streptomycin (Kijima-Tanaka et al., 2003, Khachatryan et al., 2004, Bywater et al., 2004). A similar trend would be expected in beef cattle from western Canada. However, the proportion of resistant organisms could potentially vary between livestock species and management systems; several examples of this are provided.

A study of AMR in *E. coli* isolated from healthy poultry, pigs, and beef cattle demonstrated a diverse range of the proportion of isolates resistant for each species investigated and for each antimicrobial (Kijima-Tanaka et al., 2003). In general terms broilers had more resistance than pigs which had more resistance than cattle. For example, resistance to oxytetracycline ranged from 25 and 69% for cattle and broilers respectively (Kijima-Tanaka et al., 2003). This study also reported that fluoroquinolone resistance was 10% in broilers, while in cattle and pigs fluoroquinolone resistance was detected in <1% of the isolates (Kijima-Tanaka et al., 2003).

Schroeder et al., (2002) demonstrated similar trends in their study of *E. coli* 0157 isolated from diagnostic samples collected from humans, cattle, swine, and food. Swine were found to be the most resistant species investigated in this study. Sulphamethoxazole resistance was detected in 74% of the swine isolates, tetracycline resistance was detected in 71% of the swine isolates, and cephalothin resistance was detected in 54% of the swine isolates (Schroeder et al., 2002). The type or age of the cattle was not specified, but 14% of the cattle isolates were positive for sulphamethoxazole resistance, 20% were positive for tetracycline resistance, and 3% were positive for ampicillin resistance (Schroeder et al., 2002).

Sayah et al. (2005) also reported AMR patterns obtained from domestic and wild animal fecal samples, human septage, and surface water. The animal samples were collected from beef (7 farms), dairy (7 farms), swine (5 farms), horses (2 farms), sheep (6 farms), goats, chickens (2 farms), cats, dogs, deer (2 farms), ducks, and geese. The actual number of antimicrobials present in multiple resistant isolates was highest in swine, followed by dairy, poultry, and then beef cattle (Sayah et al., 2005). The highest levels of AMR varied depending on the antimicrobial of interest and the livestock species. Companion animals had the most resistance detected to cephalothin (38%), followed by small ruminants (22%), and then cattle (21%). Swine (63%) had the most isolates resistant to tetracycline, followed by poultry (35%), and small ruminants (24%) (Sayah et al., 2005).

A study examining the point prevalence of AMR *E. coli* O157 in Saskatchewan feedlot cattle reported that of 131 isolates, 65% were resistant to at least one antimicrobial tested (Vidovic and Korber, 2006). No resistance was observed to amikacin, ampicillin, amoxicillin-clavulanic acid, ceftriaxone, ciprofloxacin, cefoxitin, gentamicin, kanamycin, nalidixic acid, trimethoprim-sulphamethoxazole, and ceftiofur (Vidovic and Korber, 2006). Sulphasoxizole and tetracycline resistance were detected in 61% and 12% of the isolates respectively (Vidovic and Korber, 2006). Chloramphenicol and streptomycin resistance was detected in 2.3% of the isolates (Vidovic and Korber, 2006).

In a longitudinal feedlot study performed in Alberta, cattle were sampled on arrival, at day 70 and again prior to slaughter. Animals were examined for vancomycin-resistant *Enterococcus faecium* (VRE) and *Enterococcus faecalis*, for *Salmonella*; quinolone or macrolide resistant thermophilic *Campylobacter*; and quinolone, aminoglycoside, or beta-lactam resistant *E. coli* (Read et al., 2004). No VRE and no *Salmonella* were detected. Low levels of ciprofloxacin, enrofloxacin, azithromycin, gentamicin, and meropenem resitance were observed in the *Campylobacter* isolates (Read et al., 2004). No ciprofloxacin resistant *E. coli* were detected, and only low levels of getamicin resistance was detected in *E. coli* (Read et al., 2004). Ampicillin resistance was detected in 15% of the animals on entry, 60% at the interim sampling, and 63% prior to slaughter (Read et al., 2004). Ampicillin resistance was associated with florfenicol use and with tetracycline in feed (Read et al., 2004). A subset of the ampicillin resistant isolates were also $bla_{\text{CMY-2}}$ positive (Read et al., 2004). The presence of the $bla_{\text{CMY-2}}$

gene was associated with therapeutic use of florfenicol, oxytetracycline and tilimicosin at entry into the feedlot (Read et al., 2004).

Two dairy cattle prevalence papers were also identified to demonstrate the range of AMR in this livestock sector. In one study, of 213 lactating dairy cows on 23 herds in Pennsylvania, *E. coli* isolates were found to be resistant to ampicillin (48%), ceftiofur (11%), florfenicol (78%), chloramphenicol (20%), spectinomycin (18%) and tetracycline (93%) (Sawant et al., 2007). Multi-drug resistance (≥3 antimicrobials) was detected in 40% of the *E. coli* isolates (Sawant et al., 2007). The most common multiple drug resistance pattern contained ampicillin, florfenicol and tetracycline; 36% of the multi-resistant isolates contained this pattern (Sawant et al., 2007). The second dairy study examined 96, 1 to 9 week old dairy calves from a single herd in Pennsylvania. AMR *E. coli* was found in 100% of the isolates (n=122) (Donaldson et al., 2006). All isolates contained both ampicillin and ceftiofur resistance. High levels of resistance were also detected for chloramphenicol (94%), florfenicol (93%), gentamicin (89%), spectinomycin (72%), tetracycline (98%) and ticarcillin (99%) (Donaldson et al., 2006). Cluster analysis indicated that 63% of the isolates belonged to one group (Donaldson et al., 2006). The bla_{CMY2} gene was found in 96% of the ceftiofur resistant isolates (Donaldson et al., 2006).

Both beef and dairy calves were included in a study from Scotland. This project examined 72 beef suckler calves and 29 dairy calves on 15 cattle farms with cases of active enteritis and 9 farms without active cases of enteritis (Gunn et al., 2003).

Between 1 and 9 animals were sampled on each farm. Ampicillin resistance was founding 84% of the isolates, apramycin resistance in 13% of the isoalates, and nalidixic acid resistance in 6% of the isolates (Gunn et al., 2003). AMR was more frequently detected in the calves with enteritis than in the controls (Gunn et al., 2003). In calves with diarrhea, 95% of the isolates were resistant to ampicillin, 22% to apramycin, and 11% to nalidixic acid (Gunn et al., 2003). In control calves, 70% of the isolates were resistant to ampicillin, 2% to apramycin, and 0% to nalidixic acid (Gunn et al., 2003).

Hoyle et al. (2006) investigated AMR on an organic beef farm in Scotland over a 28 month period. Multiple resistance was found in >44% of the isolates with ampicillin, neomycin, sulphamethoxazole, and tetracycline resistance being the most common (Hoyle et al, 2006). In all calf cohorts examined, the peak monthly prevalence for ampicillin resistance ranged from 47 to 100% (Hoyle et al, 2006). Apramycin and nalidixic acid resistant *E. coli* were not detected in any fecal samples Hoyle et al, (2006).

These papers illustrate a wide range of resistance prevalence depending on the species of livestock investigated and the antimicrobial of interest. Even within cattle there are numerous AMR patterns and frequencies. Since cattle data are often presented together (dairy, feedlot, cow, calf), or as dairy or feedlot specifically, no information was found for cow-calf herds.

2.10.4. Distribution of AMR in cattle populations

Earlier research has indicated that prevalence of resistance is not equally distributed by age (Brophy et al., 1977, Hinton et al., 1984, Hinton, 1985, Matthew et al., 1999, Khachatryn et al., 2004). Typically AMR is highest in young animals (Khachatryn et al., 2004) and declines linearly with age (Hoyle et al., 2004). This phenomenon is not fully understood, but various theories have been investigated.

Hoyle et al. (2004) demonstrated that calves preferentially lost resistant relative to susceptible bacteria as they aged. Additionally, other research has indicated that even in the absence of antimicrobials, a high prevalence of AMR could be maintained because the resistant strains had a fitness advantage in young calves but not in older animals (Khachatryan et al., 2004). The presence of these resistant *E. coli* in the absence of treatment/selective pressure could be due to fitness traits that make them better able to compete in the calf gut compared to susceptible organisms. These traits may include non-scavenging mechanisms (siderophores), increased adhesion and mechanisms that enhance colonization, reproduction, and spread (Simmons et al., 1988, Visca et al., 1991, Allen et al., 1993, Mandal et al., 2001).

2.10.5. Risk factors for AMR in calves

A recent study of commensal *E. coli* isolated from pre-weaned dairy calves on dedicated calf rearing facilities (calf ranches) and on dairy farms described many factors associated with AMR (Berge et al, 2003, Berge et al, 2005a). Farm type, animal source,

calf age, and individual treatments were important predictors of the odds of *E. coli* belonging to resistant clusters (Berge et al., 2005a). Calves 2 weeks of age and older were more likely to carry multiple antimicrobial resistant organisms than day old calves. Calves on a dedicated calf rearing facility were also more likely to be carrying multiple resistant *E. coli* than calves reared on traditional dairy farms. *E. coli* isolated from calves treated with antimicrobials within 5 days of sampling were also more likely to be multiply resistant than *E. coli* isolated from calves not exposed to antimicrobial therapy. The authors concluded that the higher levels of resistance in calves raised on calf ranches was a result of selective pressure due to antimicrobials in the milk replacers throughout the pre-weaning period. Similarly, it was concluded that systemic antimicrobial treatment within 5 days of sampling also applied selective pressure on the enteric commensal flora.

Additional work by Berge et al. (2006) investigating prophylactic and therapeutic antimicrobial administration on AMR of fecal *E. coli* in dairy calves indicated that in-feed antimicrobials were associated with higher levels of multiple AMR; that in calves not receiving in-feed antimicrobials, older calves had higher levels of multiple AMR than day-old calves; and that individual treatment with antimicrobials transiently increased resistance. Based on this and the previously mentioned studies, they concluded that the occurrence of AMR in commensal *E. coli* is dominated by selective influence.

Further work indicated that in feedlot cattle, treated animals shed a larger proportion of resistant organisms in their feces initially after therapy, but that this level declined gradually over 4 weeks (Berge et al., 2005b). Despite transient increased shedding of resistant organisms in the treated individuals, there was no effect on the shedding of resistant organisms in the untreated pen mates (Berge et al., 2005b). The authors concluded that this demonstrated that there is limited transfer of AMR bacteria from treated to untreated animals (Berge et al., 2005b).

The above feedlot study also demonstrated an interesting dynamic of AMR. The calves for the study came from two sources. While individual animal treatment records were not available, calves at neither source farm had exposure to antimicrobials in the feed prior to arrival at the feedlot, but *E. coli* isolated from calves from one source appeared more susceptible on arrival than *E. coli* isolated from calves originating from the other farm (Berge et al., 2005b). The calves from the source farm that were shedding *E. coli* with lower levels of AMR at arrival eventually did shift to higher levels of resistance over time ultimately leading to a more uniform distribution of AMR regardless of farm of origin. From this the authors hypothesized that the original source of calves may impact fecal *E. coli* patterns initially, but over time the feedlot environment dictates what patterns eventually become established.

2.10.6. How this thesis will fill in the gaps demonstrated in this review

There are very few data on AMU and AMR in beef cattle and no information on western Canadian cow-calf herds. For the few studies available, the age and type of

animal being sampled are not always defined. In order to appreciate the potential diversity of AMR in cow-calf herds, a methodical sampling approach is necessary to investigate AMR patterns in the primary age groups found in cow-calf herds. Risk factors for AMR development in cow-calf herds also need to be identified including information about the reasons for treatment and the types of antimicrobials used. To date this information is not available.

2.11. Concluding statements

AMR is a complex issue. While in the last several years great strides have been made in gaining a better understanding of the underlying genetic mechanisms for AMR, there is still much to be learned. The issue of AMU in livestock and its subsequent impact on human health will probably continue to be debated despite the growing knowledge base that is being accumulated. While this question may be a long way from being resolved any additional work investigating the development of AMR in animals and in people will help add an additional piece to the puzzle. Despite a growing body of information on AMR scientists are still struggling with grasping all the intricacies of this subject.

When it comes to AMU in livestock the knowledge gap grows exponentially. In order to be able to appreciate the true impact of livestock AMU on AMR, a much better system of collecting and reporting AMU needs to be developed. For most countries, and in the majority of species, unfortunately the reality of collecting good AMU data at this time is truly limited.

Since AMR is generally a sporadically encountered problem in therapeutic failure in veterinary medicine, it may be perceived by some as a non-issue. Unfortunately, it is not just therapeutic failure that veterinary medicine and the livestock industry need to be concerned with, but it is also the public perception of our role in the spread of AMR through "over" use of antimicrobials in the rearing of animals. Public perception and intensive scrutiny of AMU in food animal production may ultimately lead to the banning of certain antimicrobials and future limitations on the approval of specific antimicrobials for use in animals. Our current AMU practices may also lead to potential trade barriers from countries that have already banned in feed antimicrobials. Unfortunately, in Canada, despite research and surveillance efforts, there are still large gaps in our understanding of AMU and AMR. By expanding our current knowledge and by conducting research at all levels of the food chain, science can be used to help ensure that antimicrobials will remain viable and available for the health and welfare of both humans and animals.

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CHAPTER 3
ANTIMICROBIAL USE IN 203 WESTERN CANADIAN COW-CALF HERDS

3.1. Introduction

The treatment of infectious disease in food producing animals is an essential component of veterinary medicine. Antimicrobial therapy is an important tool available to producers and veterinarians and is necessary to ensure that animal health and welfare are maintained. In addition to therapeutic use, antimicrobials are also used non-therapeutically to prevent disease, for growth promotion, and to increase production efficiency.

Antimicrobial use (AMU) in food animal production is under increasing scrutiny because of reports in both the scientific literature and lay media concerning antimicrobial resistance (AMR) and the emergence of multi-resistant pathogens. Most authorities believe the association between AMU and AMR to be virtually certain (Shales et al., 1997). There is literature supporting the link between the use of antimicrobials in both people and animals and the selection for resistant bacterial populations (Sandvang et al., 1997, Tollefson et al., 1997, Barton, 1998, Levy, 1998). If a link between AMU and AMR is accepted, then the question becomes whether the populations of resistant bacteria identified in people and animals are independent or whether they comprise a common pool and pose a potential threat to both human and

animal health (Salyers and Cuevas, 1997, Barton, 1998). If human and animal bacterial populations comprise a common pool, then AMU in animals could impact the AMR in both animal and human populations.

Resistance mechanisms have been described and identified for all antimicrobials that are currently available for clinical use (McDermott et al., 2002). Research demonstrates that both veterinary and human pathogens such as *Escherichia coli* (Sidjabat et al., 2006), *Salmonella* spp. (Lopes et al., 2006), *Enterococcus* spp. (Manero et al., 2006), *Staphylococcus* spp. (Guardabassi et al., 2004, Sabour et al., 2004), and *Campylobacter* spp. (Randall et al., 2003) have acquired multiple resistant phenotypes. Options for antimicrobial therapy against disease caused by these organisms could become limited or non-existent in the near future (Levy, 1994, ASM, 1995, Gold and Moellering, 1996, Salyers and Cuevas, 1997, Levy, 1998). This concern has resulted in increased awareness about AMU and the subsequent development of AMR.

While many countries are developing surveillance systems for AMU and AMR, there is little information on which antimicrobials are used, how they are used, and in what quantities. While a few studies have provided some insight to more intensive livestock production units such as hog farms (Dunlop, 1998, Rajic, 2006), there is no information about AMU in western Canadian cow-calf herds.

Based on farm cash receipts the beef industry is the largest livestock commodity in Canada (Statistics Canada, Accessed May 18, 2007,

http://www.statcan.ca/Daily/English/07022007/d070227a.htm). The provinces of Alberta and Saskatchewan contain more than 65% of the beef cow, breeding heifer, and calf populations in Canada (Statistics Canada, Accessed July 25, 2006, http://www.40.statcan.ca/101/cst01/prim50a.htm). A better understanding of AMU patterns in this population is essential to develop a baseline and determine the need for future monitoring in the Canadian cow-calf industry.

The objective of this study was to describe the frequency of treatment with antimicrobials, common reasons for AMU, and the types of antimicrobials used in western Canadian cow-calf herds. It was beyond the scope of this project to attempt to quantify the amount of antimicrobials used in these operations.

3.2. Materials and methods

3.2.1. Background and herd selection

The herds examined in this study were part of a multifaceted survey of risk factors affecting the productivity and health of cow-calf herds in western Canada (https://www.wissa.info). Sixty-one private veterinary clinics across Alberta, Saskatchewan, and north-eastern British Columbia were approached and asked to participate. Within each practice, herds were identified and enrolled based on the selection criteria which considered herd size (>50 cows), animal identification, existing calving records, animal handling facilities, routine testing for pregnancy and for bull breeding soundness, and relationship with a local veterinary clinic. Only herds using a

winter/spring calving season were enrolled in the study. Participating herds were visited regularly by one of six veterinarians contracted by the University of Saskatchewan to collect samples and data and to monitor the quality and consistency of on-farm records.

The individual animal treatment records from January 1 to June 30, 2002 were summarized for 203 study herds. Calf and cow/heifer treatment data were investigated separately. In the first step of the analysis only calf treatment data were considered. The analysis was restricted to information collected for calves born alive between January 1 and May 31, 2002. Risk factor data were summarized for calves and their dams meeting the inclusion criteria (Table 3.1). The second step of the analysis included treatment data reported for all cows, bred heifers and yearling heifers in the herd on January 1, 2002. Cows and bred heifers with stillborn calves, non-pregnant cows and heifers were also incorporated into the total number of animals available for investigation (Table 3.2). Any cows or heifers purchased after January 1, 2002 were not included.

Available calving records for each cow/heifer included cow/heifer identification, calf identification, date of calving, single or twin birth, sex of the calf, the type of assistance provided to the cow/heifer, any post calving problems, and calving outcome (born alive, stillbirth, died later). If the calf died, the date of death was also reported. Other data recorded for each herd included: the ecological region in which the herd was located, the veterinary clinic servicing the herd, vaccination status for infectious bovine rhinotrachietis (IBR), bovine viral diarrhea virus (BVDV), and also for neonatal diarrhea (coronavirus, rotavirus, and *E. coli*). Cow/heifer body condition (BCS) was

scored (9-point scale) at the time of pregnancy diagnosis and again before or during the early part of the calving season.

3.2.2. Antimicrobial use data collection

Data on AMU were collected using individual treatment records as well as a herd level questionnaire. Producers were provided with a standardized treatment book for recording the date of treatment, animal identification, class of animal, reason for treatment, type of treatment, outcome, and other notes. A coded list was provided to help standardize the responses for class of animal, reason for treatment, type of treatment, and outcome. Animal class included: calf, cow, heifer, and bull. Scours (diarrhea), navel ill (omphalitis), pneumonia, bloat (ruminal typmpany), arthritis, pinkeye (infectious keratoconjunctivitis), coccidiosis, and other were included as possible diagnoses. Treatment type was coded as injectable antimicrobial, oral antimicrobial, oral and injectable antimicrobial, fluids, and other. Outcome options included: survived, died later, slaughtered, and unknown. A notes section allowed producers to write in any comments. Where possible the notes section was used to help further classify diagnoses or treatments recorded.

Producers could report more than one reason for or type of treatment for each treatment episode. The producer was asked to record each treatment occurrence however, animals reported as treated more than once for the same diagnosis within a seven day period were classified as having one treatment event for the purpose of analysis.

Treatment data were then summarized in two ways. To attempt to provide an estimate of treatment intensity per herd for the period of January 1 to June 30, 2002, a count of total treatment events per herd was determined. This was calculated separately for both cows/heifers and calves and was reported as the number of treatment events per every 100 animals at risk. Animals at risk included the total number of calves or cows/heifers in the study for this time period. Treatment occurrence was also summarized separately for calves and cows/heifers as risk or cumulative incidence. This was calculated as the number of calves (or cows/heifers) that were reported as ever having been treated as a percentage of the number of calves (or cows/heifers) in the herd at risk of treatment during the study period.

The individual animal records did not consistently include information on the type of AMU for treatment, therefore, a questionnaire was developed to identify the types of antimicrobial products most commonly used on each cow-calf farm. Herd owners were asked about the frequency of use for sulphonamides, tetracylcines/oxytetracyclines, trimethoprim/sulphadiazine, and penicillins. Antimicrobials that did not fall into these broad categories were classified as “other”. Lists of common trade names were provided under each group to simplify the selection of the appropriate drug by the producer. Producers were asked to report separately the number of treatments for both cows and calves for each drug category listed above. The number of treatments for each category was coded as follows: l to 3 animals treated, 4 to 10 animals treated, and greater than 10 animals treated.

The quality of the treatment records were assessed at the end of the study. The veterinarians responsible for data collection and entry were asked for a crude subjective and comparative assessment of the quality of the data. They classified the data for each herd into one of the following categories: excellent, good or satisfactory, and less than satisfactory. Herd owner compliance in completing treatment records was also evaluated by considering the relative frequency of calf mortality in the herds that did not report any treatments. Complete calf mortality records were available for comparison from the baseline productivity study. The plausibility of no reported treatments was assessed when compared to the percent calf death loss in each herd.

3.2.3. Statistical analysis

All data were entered into a computerized database (Microsoft® Office Access 2000, Microsoft Corporation). Descriptive analyses were completed and variables were recoded as necessary for statistical modeling using commercially available software programs (SPSS 11.0 for Windows, SPSS Inc., Chicago, Illinois).

3.2.3.1. Mixed models for discrete data

Factors affecting the occurrence of treatment, a class variable with two levels (treated or not treated), were examined in both cows/heifers and calves using mixed models with a binomial distribution and logit link function. The calculations were performed using penalized quasi-likelihood estimates (2^{nd} order PQL) (MLwiN version

2.0, Centre for Multilevel Modelling, Institute of Education, London, UK). The strength of the association between outcome and exposure was reported as an odds ratio (OR) with 95% confidence intervals.

A null model (intercept only) was created for each outcome variable. Random intercepts were examined to assess degree of clustering for treatments reported within herd, veterinary clinic, and ecological region (ecoregion). Ecoregion is a geographical delineation characterized by regional ecological factors such as vegetation, soil, climate, water and fauna (Wiken, 1986). Within-herd clustering was accounted for as a random intercept in all models. The importance of veterinary clinic and ecoregion as random effects were considered for inclusion in the final model if the variance estimate for the random effect was larger than its standard error. Models were checked for the presence of extra-binomial variation, but extra-binomial parameters in the range of 0.9 to 1.0 were reset at 1.0 (binomial variation).

Data from the null models were used to estimate the intra-class (i.e., intra-herd) correlation coefficient ($\rho=\sigma^2_h/(\sigma^2_h+\pi^2/3)$) to measure clustering of each outcome within herd. The null models were also used to generate population-average estimates of the risk of calf and cow/heifer treatment using the formulas $\beta^{PA}\approx\beta^{SS}/\sqrt{(1+0.346\ \sigma^2_h)}$ (Dohoo et al., 2003).

The unconditional associations between each of the potential risk factors (Table 3.1 for calves and Table 3.2 for cows/heifers) and the odds of treatment were examined. All

potentially important risk factors ($P \leq 0.25$) were identified and a final model was then developed using backwards stepwise elimination.

Any potential risk factors where $P < 0.05$ or that were acting as important confounders (removal of the potential risk factor from the model changes the effect estimate for the exposure by≥20%) were retained in the final model. After establishing the main effects model, biologically reasonable first order interaction terms were tested if two or more variables ($P < 0.05$) were retained in the final model. The adequacy of all models was evaluated using plots of residuals to check that all assumptions had been met as appropriate.

Associations between calf treatment and mortality were investigated using generalized estimating equations (SAS v.8.2 for Windows (PROC GENMOD); SAS Institute, Cary, North Carolina, USA). The number of calves with any treatment (numerator) as a proportion of the total number of calves in the herd (denominator) was the outcome of interest. The predictor variable, percent calf mortality, was categorized into quartiles (<2%, 2-5.9%, 6-14.9% and >15% mortality) with mortality >15% as the reference. The model specifications included a binomial distribution, logit link function, repeated statement with subject equal to herd, and an exchangeable correlation structure.

3.3. Results

3.3.1. Farm and animal information

Records from 203 herds across Alberta (146), Saskatchewan (53), and northern British Columbia (4) were included. On January 1, 2002, herd size ranged from to 53 to 481 mature/breeding females, with a median herd size of 154. Of the 203 included in the study, 169 herds (83.3%) had between 100 and 400 mature/breeding females.

3.3.1.1. Calf population

Between January 1 and May 31, 2002, 28,573 calves were born alive; the majority of calves were born in March and April (64%; 18,285/28,573). Most calves born alive were unassisted; 4.4% of live births were twins (Table 3.1). About half of the dams were vaccinated for IBR/BVDV prior to breeding in 2001, while about one third of the dams received some type of vaccination to prevent neonatal calf bacterial or viral diarrhea (Table 3.1). Most cows had a BCS of 5 or higher on a 9-point scale at pregnancy testing and again at calving (Table 3.1).

3.3.1.2. Cow and heifer population

There were 36,634 cows and heifers reported in study herds on January 1, 2002. This number included all cows/heifers that had calves born alive from January 1 to May 31, 2002, cows/heifers that had abortions or stillborn calves during this period, and any

non-pregnant cows/heifers and replacement heifers. The majority of cows were between 4 and 10 years of age (Table 3.2). Dystocias were reported in <10% of cows/heifers, and <1% of cows/heifers had post partum complications such as retained placentas, prolapses, or metritis (Table 3.2).

3.3.2. Individual animal records of treatment and diagnosis

3.3.2.1. Individual calf treatment records

Herd owners reported treating 13.5% (95% CI; 10.7 to 17.0%) of the calves at least once between January 1 and June 30, 2002. The median age of calves at the time of their first treatment was 11 (range, 0-141) days of age and 58.6% (2171/3702) of the treated calves were between birth and 14 days of age. The median percent of calves ever treated per farm was 6.5% (range, 0-100%). After accounting for the records where an individual calf was treated more than once, the median number of treatment events per farm was 6.8 (range 0-104) for every 100 calves at risk.

The most commonly recorded calf treatment was antimicrobial injection (Table 3.3). Injectable antimicrobials were used in 56 (27.6%) herds on <1% of the calves, in 70 (34.5%) herds on 1-5% of the calves, in 56 (27.6%) herds on 5-15% of the calves, and in 23 (11.3%) herds on >15% of the calves. Four herd owners treated 50-80% of the calves and 3 herd owners treated all of their calves at least once. The maximum number of times a calf was treated with injectable antimicrobials was 14 (median, 1; range, 0-14).

The second most commonly reported protocol for calves included treatment with both oral and injectable antimicrobials at the same time (Table 3.3). Oral and injectable antimicrobials were used on <1% of the calves in 130 (64.0%) herds, on 1-5% of the calves in 40 (19.7%) herds, on 5-15% of the calves in 23 (11.3%) herds, and on >15% of the calves in 10 (4.9%) herds (maximum, 42.3%).

Oral antimicrobials alone were used in <2% of calves (Table 3.3). One hundred and forty-four (70.9%) herd owners treated <1% of their calves with oral antimicrobials, 40 (19.7%) treated 1-5% of the calves, 13 (6.4%) treated 5-15% of the calves, and 6 (2.9%) treated between 15-51% of their calves with oral antimicrobials.

3.3.2.2. Individual calf records of diagnoses

Diarrhea was the most commonly reported reason for treatment in calves and was diagnosed in over 5% of the calves and on 60% of the farms (Table 3.4). The percent of calves treated per farm ranged from 0 to 89.0% (median, 4.3%). Of the 203 herds/producers, 145 (71.4%) producers treated 0-5% of their calves for diarrhea, 20 (9.9%) producers treated 5-10%, 25 (12.3%) producers treated 10-20%, and 13 (6.4%) producers treated >20% of their calves for diarrhea.

The next most common reason for treatment was pneumonia (Table 3.4). Just over 2% of the calves were diagnosed with pneumonia, and calf pneumonia was reported as a reason for treatment on 50% of the farms (Table 3.4). Pneumonia treatment rates per

farm ranged from 0-52.3% (median, 2.0). One hundred and twenty-six (62.1%) producers treated ≤1% of their calves, 51 (25.1%) producers treated 1-5% of their calves, 20 (9.9%) producers treated 5-15% of their calves, and 6 (2.9%) producers treated >15% of their calves for pneumonia.

Treatment and prevention of omphalitis (navel infection) made up the third most common recorded reason for treatment (Table 3.4). Four producers treated between 65 and 100% of their calves prophylactically for omphalitis.

3.3.2.3. Individual cow treatment records

Between January 1 and June 30, 2002, 2.7% (95% CI; 2.2 to 3.4%) of the cows and heifers were treated at least once. The median percent of cows/heifers ever treated per farm was 0.9% (range 0-14.7%). Since the majority of cows/heifers were only treated once during this time period the number of treatment events per 100 cows/heifers at risk was also 0.9 (range 0-14.7).

The most commonly reported treatments in cows and replacement heifers were with injectable antimicrobials (Table 3.5). Very few cows or heifers were reported to receive either oral antimicrobials or oral and injectable antimicrobials together (Table 3.3). Oral treatments were only given in 2 (1.0%) herds and to <3% of the cows in these herds; whereas, oral and injectable treatments were given in 5 (2.5%) herds to <1% of cows. One hundred and eleven (54.7%) producers treated <1% of their cows with injectable

antimicrobials, 69 (34.0%) producers treated 1-5% of their cows, and 23 (11.3%) producers treated >5% of their cows with injectable antimicrobials.

Treatments other than antimicrobials were a more commonly reported for cows and heifers than for calves (Table 3.3). Treatments categorized as "other" were given on 46 (22.7%) herds to 0.2-5% of the cows. Other treatments included non-antimicrobial treatments such as mineral oil or other products for gastrointestinal disorders. The only antimicrobial treatment included in the other category was intra-mammary treatments for mastitis. Intramammary products were used on 3.0% of the farms (Table3.6).

3.3.2.4. Individual cow records of diagnoses

Metritis, interdigital necrobacillosis (footrot), and retained placenta were the most commonly reported reasons for treatment of cows and heifers (Table 3.5). Metritis was diagnosed and treated in <1% of the animals in 182 (89.7%) herds, in 1-2% of the animals in 9 (4.4%) herds, and in >2% of the animals in 12 (5.9%) herds. One hundred seventy-four (85.7%) herd owners treated <1% of their cows, and 29 (14.3%) of herd owners treated 1-5% of their cows for interdigital necrobacillosis. Retained placentas were diagnosed and treated in <1% of the animals in 173 (85.2%) herds, in 1-2% of the animals in 20 (9.8%) herds, and in 2% of the animals in 10 (4.9%) herds.

3.3.3. Antimicrobial use

The most commonly reported antimicrobials used in calves included (Table 3.6): oral sulphonamides, florfenicol injectable, and long acting injectable oxytetracycline. Long acting oxytetracycline injectable, benzathine/procaine penicillin G, and procaine penicillin G injectable were the antimicrobials most commonly reported to have been used in the cows and replacement heifers (Table 3.7). When all AMU was summarized for each herd, the most commonly reported antimicrobials were oxytetracycline, penicillin, and florfenicol (Table 3.8).

Ionophores were used in the feed of cows or heifers in 28.6% (58/203) of the herds. Of the herds using ionophores 86.2% (50/58) were including ionophores in both the cow and heifer rations, 10.3% (6/58) were including ionophores in heifer ration only and 3.4% (2/58) were including ionophores in the cow ration only. Reason for use was not clearly specified.

Specific treatment information was investigated for two antimicrobials of interest, enrofloxacin and florfenicol. Enrofloxacin was reported as used on 287 calves in 8 herds. One herd reported treating 172 of 191 (90.1%) calves with a combination of enrofloxacin and sulbactum-ampicillin. For all calves treated with enrofloxacin, the recorded reason for treatment was scours. Florfenicol was listed specifically in the notes section for the treatment records of 92 calves on 17 farms. Of these 92 calves, 41 (44.6%) were treated for diarrhea, 31 (33.7%) for omphalitis, 5 (5.5%) for pneumonia, 1 (1.1%) for arthritis, and 14 (15.2%) for other reasons.

3.3.4. Effect of herd, veterinary clinic, ecoregion, and other risk factors on reported treatment practices for calves and cows

Neither location of the herd by ecoregion or accounting for differences between referring veterinary clinics explained a substantial part of the variation in reported treatment practices for either calves or cows. However, treatment practices were clustered within herd (calf treatment ρ=0.21, cow treatment ρ=0.20).

Calf gender, the need for assistance at parturition, and the percent of cows/heifers treated in the herd were unconditionally associated with the odds of a calf having been reported as being treated by the herd owner (Table 3.9). After accounting for other variables in the model, male calves were at higher risk than female calves to have been treated, and calves that had a history of intervention during calving were more likely to have been treated than calves that were born unassisted (Table 3.10). Also, for every 1.0% increase in cow/heifer treatment the odds of calf being treated increased by 1.2 times (Table 3.10).

Initial analysis of the heifer and cow data demonstrated that yearling heifers were 0.2 times (95% CI 0.2-0.3, *P*=0.0001) as likely to be treated than all other breeding females. Other risk factors such as breed, precipitation, and body condition score, did not significantly contribute to the odds of a cow or heifer being treated.

Assessment of pregnant cow or heifer vaccination status and calving related factors was completed only for mature females with calving records in 2002 (Table 3.11). After accounting for other risk factors, cows or first-calf heifers that had a problem post calving such as a prolapse, retained fetal membranes, or metritis were more likely to be treated than cows or heifers that did not have a problem post calving (Table 3.12). Also, cows or heifers that needed assistance at calving were more likely to be treated than cows or heifers that did not.

3.3.5. Assessment of the quality of treatment records

The veterinarians responsible for data collection and entry subjectively rated 39% of the herd treatment records as excellent, 41% as good or satisfactory, and 20% as less than satisfactory. Herd owner compliance in recording these data was also investigated by comparing herd calf mortality and treatment rates in the 28 (13.7%) herds that had no reported treatments. Of these herds, 4 had no calf mortality, 10 had <2% calf mortality, 8 had 2-5% calf mortality, 4 had 5-10% calf mortality and 2 had >10% calf mortality. The risk of calf mortality was not associated with the proportion of calves treated in the study herds (P=0.6).

3.4. Discussion

This study is the first documentation of AMU and rationale for treatment in extensively managed cow-calf herds in western Canada. At least 86% of herd owners treated one or more calves or cows and heifers during the study period; however, less

than 15% of both calves and cows/heifers were reported as treated. The relatively small proportion of treated animals is consistent with the finding that the primary reasons for antimicrobial use in cow-calf operations were for individual therapeutic uses rather than prophylaxis, metaphylaxis, or growth promotion. In contrast, between 75-90% of all dairy cattle receive prophylactic antimicrobials to prevent mastitis (Sishco et al., 1993, USDA, 2003). Depending upon the size of the feedlot, the type of cattle placed and bovine respiratory disease risk designation, anywhere between 16-19% of feedlot cattle in the United States (USDA, 1999) and 20-50% of feedlot animals in Canada receive prophylactic injectable antimicrobials on arrival for the control of bovine respiratory disease (Radostits OM, 2001; personal communication with Calvin Booker, FHMS, February 22, 2007).

Although some oral antimicrobials were used, injectable formulations were the most commonly reported method of antimicrobial administration on cow-calf farms. Only a small number of herds used feed ionophores. This varies from feedlot or swine operations where in feed use plays a larger role in antimicrobial delivery (McEwen and Fedorka-Cray, 2002, Rajic, 2006). The most commonly used products in cows were long acting injectable oxytetracyclines and penicillins. Injectable and oral sulphonamides, injectable florfenicol, and injectable oxytetracyclines were the primary drugs selected for treatment of calves.

A Michigan study of AMU in cattle reported slightly different results. The AMU information in this study was collected by questionnaire and pertained to treatment

practices within the previous 60 days (Sayah et al., 2005). No beef cattle (n=89 beef cattle on 7 farms) in this study were treated with trimethoprim/sulphamethoxazole, tetracycline, or streptomycin (Sayah et al., 2005). The most common treatment was with sulphamethazine (55.7%) and chlortetracycline (55.7%), followed by tilmicosin (27.9%), oxytetracycline (16.5%), penicillin (2.5%), enrofloxacin (2.5%), and ceftiofur (1.3%). There are two major distinctions between the Michigan study and the current study. First, the Michigan study reported AMU for all beef cattle without differentiating between feedlot and cow-calf herds. Some of the differences in the types of drugs reported as used could be a result of this reporting structure. Second, the Michigan study only looked at AMU in the 60 days prior to the questionnaire administration in a limited number of animals while the current study looked at AMU over a 6 month period in a much larger number of animals.

Two unpublished studies from Ontario also provide further insight into AMU in beef cattle. The first study looked at 16 feedlots and 13 cow-calf farms (Bair and McEwen, 2001). Penicillin was used on 45% of the farms, florfenicol on 35%, ceftiofur on 17%, oxytetracycline in 22%, tilmicosin on 80%, and sulbactam-ampicillin on 11%. No in feed AMU was reported for the cow-calf herds other than the inclusion of an ionophore (9/13 farms). In the second Ontario study, 587 (341 cow-calf and 106 feedlot) producers were surveyed on their attitudes about AMU (Powell and Powell, 2001). The producers surveyed reported using the following injectable antimicrobials: oxytetracycline (50.0% of herds), penicillin (48.5%), tilmicosin (78.0%), trimethoprim-sulphadoxine (23.0%) and florfenicol (14.5%) (Powell and Powell, 2001). As with the above mentioned

Michigan study, the reporting structure of the two Ontario studies does not permit differentiation between what was used in cow-calf herds and what was used in feedlots.

Extra-label drug use was reported in some cow-calf herds in the current study. In Canada, florfenicol is labeled for bovine respiratory disease and for the treatment of interdigital phlegmon (Compendium of Veterinary Products, 2003), but the individual animal treatment notes indicate that it was also used in an extra-label manner in calves for diarrhea and omphalitis. Extra-label use of fluoroquinolones and cephalosporins was also reported. Powell and Powell (2001) also reported off label use of enrofloxacin in their survey of Ontario beef producers. At the time of these studies, enrofloxacin was not readily obtainable by cattle producers because in 2002 there was only a small animal formulation available in Canada. Recently, a cattle formulation has been approved for use in Canada for the treatment of bovine respiratory disease (Compendium of Veterinary Products, 2003). Follow up studies to see how AMU patterns might have changed with a change in product availability are needed.

In addition to describing use patterns, this study identified factors associated with the reported frequency of calf treatment. Calves that were assisted during birth were more likely to be reported as treated. Sanderson and Dargatz (2000) also reported that increasing incidence of dystocia in a herd was associated with increased morbidity. Dystocia can lead to decreased vigor, hypoxia and acid-base disturbances (Bellows et al., 1987). Another potentially related finding was that male calves were more likely to be treated than female calves. This could be because male calves are often larger than

females (Bellows et al., 1987). Larger calves are more likely to experience delayed parturition and increased fetal stress. This could result in reduced vigor; potentially negatively affecting passive transfer and calf health. Calves were also more likely to be treated in herds where more cows/heifers were treated. This finding might reflect an increased likelihood of exposure to disease on these farms because of management or other factors, or it may reflect an increased tendency of these producers to administer and report treatment.

Yearling heifers were less likely to be treated than cows. This is probably because yearling heifers have not yet entered the breeding herd and, therefore, were not subject to the primary risk factors for treatment identified within the breeding herd. Risk factors for cow/bred heifer treatment included assistance at calving and post calving problems such as a prolapse, retained fetal membranes, and metritis. The odds that a cow or bred heifer would be treated if she had any one of the above post calving conditions were substantially increased over that of one with no problem at calving. Assistance at calving was also a risk factor for treatment. Cows or bred heifers that required any manipulation or traction on the fetus at the time of calving or caesarian sections were at much higher risk for being treated than those that calved unassisted.

Because cows and bred heifers were more likely to be treated if there was assistance at calving, further investigation is needed into whether producers are providing treatment prophylactically because of the intervention or it they are treating for an

actual condition, either related to parturition or for some other reason. This distinction is not entirely clear from the data available.

To minimize the need for treatment, producers should work to decrease the need for calving assistance and post partum complications through bull selection, management and selection of replacement heifers, and appropriate dystocia intervention (Chenowith and Sanderson, 2001). These practices may also help minimize post partum uterine prolapses through reduction of dystocias. Increasing the awareness of producers about when and why to treat is also essential. Basic manipulations or pulling of calves should not generally require antimicrobial treatment of either the dam or the calf. Also, providing adequate nutrition will help avoid post partum problems such as retained fetal membranes (USDA, 1996) as well as contributing to over all cow/heifer and calf health.

Calves were more likely to be reported as treated than cows/heifers in this study and the primary reason reported for calf treatment was diarrhea. Diarrhea was also the most commonly reported illness in beef calves in the United States (USDA, 1997) and in a survey of beef producers in Ontario (Powell and Powell, 2001). In the current study the primary reported reason for treatment of cows/heifers was metritis followed closely by interdigital necrobacillosis; whereas in the United States pinkeye and interdigital necrobacillosis were listed as the two primary disease conditions reported among breeding females (USDA, 1997). The current study only looked at treatment from January to June whereas the NAHMS study questions spanned the entire year. Infectious bovine keratoconjunctivitis (IKC) is more common in the summer months.

Although, there is a difference in the primary reason for cow/heifer treatment between western Canada and the United States both studies did report a relatively low occurrence of disease and treatment in breeding females.

A higher proportion of animals were reported as treated by owners participating in the USDA's National Animal Health Monitoring System (1997) than in the current study. This may in part be due to management, animal genetic and climatic differences between western Canada and the United States, but it may also relate to number of herds enrolled, and data collection methodology differences in each study. The National Animal Health Monitoring System's (NAHMS) beef '97 study data were collected from 2,713 producers via a questionnaire administered on farm from December 30 through February 3, 1997 (USDA, 1997). Another factor that could contribute to treatment differences is that all of the herds in the current study had more than 100 total cows and calves; whereas, less than 60% of the NAHMS herds had a herd size greater than 100. Herd size and related management factors could also account for some of the differences in treatment rates between the current study and the NAHMS study.

The NAHMS data were limited to the herd as no data were collected at the individual animal level. In contrast to the NAHMS study both individual animal records and questionnaires were used in the current cow-calf study. Tracking individual animal diagnoses and treatments may be less subject to recall bias than using a questionnaire, but relying on the completeness of individual animal treatment records may lead to underreporting.

From the Agriculture census in 2001, Statistics Canada reported that the average beef herd size for Canada was 53 cows. In Saskatchewan and Alberta the average herd sizes were 58 and 74 beef cows per herd with 15 and 20 replacement heifers respectively. The average study herd size of 180 is larger than that reported by Statistics Canada. Because herds were enrolled in the larger productivity study based on their ability to provide the required data, these herds probably represent some of the more progressive, commercially viable, and intensively managed herds in western Canada. The herds providing data for this study therefore represent AMU in this sector of the industry. AMU may be different in the few very large cow-calf herds that receive little or no treatment interventions or the very small herds present on some mixed or hobby farms.

Future studies need to focus on determining the amount of each antimicrobial used to more accurately assess animal AMU exposure. However, AMU data are difficult to collect and report for several reasons. Complete and accurate farm based AMU records are difficult to obtain. Capture of use information can be demanding for producers especially during busy times and with limited resources. Under reporting is potentially a problem since producers are busy with day to day operations on the farm and, therefore, record keeping is often a relatively low priority and subsequently treatment records may be forgotten. Dunlop et al. (1998) reported a 35% under-reporting rate for AMU recorded by swine producers when compared to inventory and disappearance data collected by the researchers. A preliminary report of AMU in the Ontario beef industry estimated average under reporting of AMU on farm was 40% (Bair and McEwen,

2001). This estimate was based on treatment diaries and accounting of drug disappearance in both feedlots and cow-calf herds. Under reporting for cow-calf herds ranged from 1-86% with a mean of 24% (Bair and McEwen, 2001).

It is difficult to fully evaluate the degree of under reporting in this cow-calf project. From the crude subjective and comparative assessment of the quality of the data, at least 20% of the herds had less than satisfactory treatment records. When combined with information on the calf mortality and the proportion of herds reporting no treatments there is further evidence that there was under reporting by some herds. It would be unlikely to have herds with greater than 5% calf death losses with no treatments. A true estimate can not be made about the degree of under reporting in these herds. However, with 20% of the herds having less than satisfactory treatment records it is likely that these herds were under reporting treatment events. Additionally, for the herds with no treatments and greater than 5% death losses there are also potentially missing treatment records.

The second problem with AMU data collection and reporting is that there is no widely accepted method for quantifying AMU (Singer et al., 2006). Use can be reported in many ways including, but not limited to, total volume of drug in kilograms, defined daily doses (DDD) (Jensen et al. 2006), animal daily doses (ADD) (Jensen et al., 2006), or as animal units per treatment days (Dunlop et al, 1998). While each of the above methods attempt to capture the true exposure of an animal to a drug limitations still exist (Jensen et al., 2006). Although there are problems with data capture and reporting,

there is international interest in developing surveillance systems for AMU and AMR potentially including farm level or aggregate level of AMU (Rosdahl and Pederson, 1998, Nicholls et al., 2001 and WHO 2001).

For farm-based AMU data to be valuable in surveillance studies, the following pieces of information need to be collected: total amount of antimicrobial used, indication for treatment, route of administration, and dose and duration (Singer et al., 2006). While several of these criteria were met by the individual animal records collected as part of the current study, detailed information on which antimicrobials were used to treat specific conditions and the dose used were not consistently reported. While an attempt was made to collect more information on specific drug use, these data were potentially subject to recall bias since the questionnaire was administered at the end of the calving season and relied on producer accounts of the number of animals treated with each class of antimicrobial.

Despite the limitations, this study does provide the first available documentation of the proportion of calves and cows/heifers reported as treated during the calving season and the types of conditions most often treated in western Canadian herds. The study also provides some initial information about AMU practices in these herds which can be used to help address issues such as extra-label drug use, prophylactic treatment of entire calf crops, and the importance of minimizing dystocia in reducing the need for treatment of either cows/heifers or calves.

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Table 3.1. Summary of animal and herd-level risk factors for calf treatment and mortality during the 2002 calving season (n=28,573; N=203). Data pertains to calves born alive January 1 to May 31, 2002 and their dams

Risk factor	Proportion of cows with attribute	Proportion of herds with at least one cow with attribute
Ecoregion		
1. Aspen Parkland	26.3%(7516)	26.1%(53)
2. Boreal Transition	10.7%(3047)	10.8%(22)
3. Fescue Grassland	13.2%(3773)	12.3%(25)
4. Mixed Grassland	14.7%(4202)	14.3%(29)
5. Moist Mixed Grassland	12.5%(3566)	12.8%(26)
6. Northern Continental Divide	4.2%(1193)	3.9%(8)
7. Peace Lowland	9.5%(2710)	12.3%(25)
8. Western Alberta Upland	4.7%(1338)	3.9%(8)
9. Western Boreal	4.3%(1228)	3.5%(7)
Vaccinated for BVDV/IBR prebreeding 2001		
1. Live vaccine	41.6%(11896)	41.9%(85)
2. Inactivated vaccine	15.7%(4491)	17.2%(35)
3. No vaccine	4.1%(1177)	3.5%(7)
4. Not reported	38.5%(11009)	37.4%(76)
Heifers vaccinated for diarrhea (E. coli) precalving 2002	38.5%(1828/4748)	35.3%(65/184)
Cows vaccinated for diarrhea (E. coli) precalving 2002	32.5%(7737/23825)	32.0%(65/203)
Heifers vaccinated for diarrhea (viral) precalving 2002	37.6%(1785/4748)	34.2%(63/184)
Cows vaccinated for diarrhea (viral) precalving 2002	31.6%(7518/23825)	31.0%(63/203)
BCS pre-calving <5 (9-point scale)	5.7%(1636)	70.0%(142)
BCS at pregnancy testing <5 (9-point scale)	8.3%(2357)	82.8%(168)
Cow born on farm and not purchased	66.5%(18997)	92.1%(187)
Twin births	4.4%(1256)	87.7%(178)
Calf gender		
1. Male	50.8%(14526)	100.0%(203)
2. Female	47.0%(13416)	100.0%(203)
3. Not recorded	2.2%(631)	55.7%(113)
No cow problem other than dystocia	98.8%(28242)	100.0%(203)
Prolapse	0.2%(50)	21.2%(43)
Retained fetal membrane (RFM)	1.0%(275)	40.4%(82)
Metritis	0.02%(6)	2.5%(5)
Calving assistance reported		
1. No assistance	92.0%(26291)	100.0%(203)
2. Easy pull	4.9%(1395)	87.2%(177)
3. Hard pull	1.7%(474)	68.0%(138)
4. Malpresentation	1.0%(285)	54.7%(111)
5. Caesarean section surgery	0.5%(128)	34.0%(69)
Calving Month		
1. January 2002	8.0%(2271)	48.3%(98)
2. February 2002	21.4%(6115)	74.9%(152)
3. March 2002	38.9%(11109)	97.0%(197)
4. April 2002	25.1%(7176)	96.1%(195)
5. May 2002	6.7%(1902)	81.3%(165)
Predominant breed type		
1. British	43.2%(12353)	82.8%(168)
2. Continental	47.9%(13692)	78.8%(160)
3. Cross	7.9%(2270)	31.5%(64)
4. No record	0.9%(258)	16.8%(34)
Age category		
1. Yearling heifer (born 2001)	0.1%(32)	10.3%(21)
2. 2 year old heifer (born 2000)	16.6%(4748)	90.6%(184)
3. 3 year old cow (born 1999)	15.7%(4497)	96.6%(196)
4. Mature cow (born 1993 to 1998)	53.2%(15206)	100.0%(203)
5. Old cow (born 1991 or earlier)	11.6%(3300)	94.1%(191)
6. No record of age	2.8%(790)	20.7%(42)

Table 3.2. Summary of animal and herd-level risk factors for cow or heifer treatment and mortality during the 2002 calving season (n=36,634; N=203)[a]

Risk factor	Proportion of cows with attribute	Proportion of herds with at least one cow with attribute
Ecoregion		
1. Aspen Parkland	24.8%(9086)	26.1%(53)
2. Boreal Transition	10.5%(3838)	10.8%(22)
3. Fescue Grassland	13.3%(4884)	12.3%(25)
4. Mixed Grassland	14.4%(5283)	14.3%(29)
5. Moist Mixed Grassland	13.2%(4822)	12.8%(26)
6. Northern Continental Divide	4.5%(1648)	3.9%(8)
7. Peace Lowlad	10.1%(3720)	12.3%(25)
8. Western Alberta Upland	4.6%(1668)	3.9%(8)
9. Western Boreal	4.6%(1685)	3.5%(7)
Vaccinated for BVDV/IBR prebreeding 2001		
1. Live vaccine	15.8%(5772)	17.2%(35)
2. Inactivated vaccine	41.8%(15317)	41.9%(85)
3. No vaccine	38.3%(14033)	37.4%(76)
4. Not reported	4.1%(1512)	3.5%(7)
Heifers vaccinated for diarrhea (E.coli) precalving 2002	33.6%(1749/5207)	35.1%(65/185)
Cows vaccinated for diarrhea (E. coli) precalving 2002	34.9%(9092/26040)	34.5%(70/203)
Heifers vaccinated for diarrhea (viral) precalving 2002	37.8%(1969/5207)	35.1%(65/185)
Cows vaccinated for diarrhea (viral) precalving 2002	28.9%(7518/26040)	31.0%(63/203)
BCS pre-calving <5 (9-point scale)	6.0%(1740/29173)	71.9%(146)
BCS at pregnancy testing <5 (9-point scale)	8.4%(3063/36464)	77.3%(157)
Cow born on farm and not purchased	51.8%(18997)	92.1%(187)
No cow problem post partum	99.0%(30901/31247)	100.0%(203)
Prolapse	0.2%(57/31247)	21.2%(43)
Retained fetal membrane	0.9%(281/31247)	40.4%(82)
Metritis	0.03%(8/31247)	2.5%(5)
Calving assistance reported		
1. No assistance	93.9%(29337/31247)	100.0%(203)
2. Easy pull	4.4%(1392/31247)	87.2%(177)
3. Hard pull	1.7%(538/31247)	68.0%(138)
4. Malpresentation	1.2%(367/31247)	54.7%(111)
5. Caesarean section surgery	0.5%(151/31247)	34.0%(69)
Predominant breed type		
1. British	43.0%(15755)	83.7%(170)
2. Continental	46.6%(17075)	80.3%(163)
3. Cross	9.4%(3424)	36.0%(73)
4. No record	1.0%(380)	16.8%(34)
Age category		
1. Yearling heifer (born 2001)	14.7%(5387)	84.2%(171)
2. 2 year old heifer (born 2000)	14.2%(5207)	91.1%(185)
3. 3 year old cow (born 1999)	13.2%(4837)	96.1%(195)
4. Mature cow (born 1993 to 1998)	44.7%(16364)	99.5%(202)
5. Old cow (born 1991 or earlier)	10.0%(3648)	95.6%(194)
6. No record of age	3.3%(1191)	22.2%(45)

[a]Data pertains to all adult females in the herd as of January 1, 2002, except for attributes that are specific to bred cows and heifers. Denominators are provided in instances where data are only applicable to a sub-set of animals. Calving information includes stillbirths, abortions and live calves. Not all animals had BCS available.

Table 3.3. Type of treatment for calves (n=28,573) and cows/heifers (n=36,634) at the animal and herd level (N=203) between January 1 and June 30, 2002[a]

Treatment	Number (%) of calves	# (%) herds reporting calf treatment	Number(%) of cows/heifers	# (%) herds reporting cow treatment
Fluids	123 (0.4)	54 (26.6)	1 (0.003)	1 (0.5)
Injectable antimicrobials	2400 (8.4)	162 (79.8)	658 (1.8)	123 (60.6)
Oral antimicrobials	512 (1.8)	80 (39.4)	3 (0.008)	2 (1.0)
Oral and injectable antimicrobials	852 (3.0)	93 (45.8)	6 (0.02)	5 (2.5)
Other treatment[b]	173 (0.6)	61 (30.0)	91 (0.3)	46 (22.7)

[a]Any individual animal may have been treated with more than one type of treatment
[b]Other treatment includes; treatment with mineral oil, intramammary infusions, vitamin injections, etc.

Table 3.4. Diagnoses recorded from January 1 to June 30, 2002 summarized at the individual calf and herd level (n=28,573, N=203).[a]

Diagnosis	Number of calves affected	% of all calves	Number of herds	% of herds
Diarrhea	1648	5.77	129	63.6
Pneumonia	625	2.19	103	50.7
Prophylactic tx for navel infections	529	1.85	4	2.0
Not recorded	355	1.24	67	33.0
Omphalitis	300	1.05	87	42.9
Fever, depression, not doing well	139	0.49	44	21.7
Coccidiosis	114	0.4	35	17.2
Prophylactic tx at castration	45	0.16	3	1.5
White muscle dz suspected	32	0.11	6	3.0
Ruminal tympany	28	0.1	23	11.3
Weak	25	0.09	14	6.9
Interdigital necrobacillosis	27	0.09	20	9.9
Infectious arthritis	20	0.07	13	6.4
Lameness	20	0.07	14	6.9
GI/ torsion/ulcers	21	0.07	15	7.4
Diptheria	13	0.05	10	4.9
Ear/eye infections	13	0.05	11	5.4
Hypothermia	14	0.05	12	5.9
Abscess	12	0.04	8	3.9
Dehydrated	8	0.03	8	3.9
Infectious keratoconjunctivitis	8	0.03	5	2.5
Surgery	9	0.03	8	3.9
Meningitis	5	0.02	5	2.5
Broken bones	7	0.02	7	3.5
Cuts/wounds	2	0.01	2	1.0
Prolapsed rectum	2	0.01	2	1.0
Blind	2	0.01	2	1.0
Predator Attack	2	0.01	1	0.5
Dystocia (hard pull)	3	0.01	3	1.5
Septecemia	3	0.01	3	1.5
Malnutrion	4	0.01	3	1.5
Arthritis	1	0.003	1	0.5

[a]Individual calves may have had more than one diagnoses.

Table 3.5. Diagnoses made from January 1 to June 30, 2002 summarized at the individual cow/heifer and herd level. (n=36,634, N=203) [a]

Diagnoses	Number of cows/heifers affected	% of total cows and heifers	Number of herds	% of herds
Metritis	145	0.4	52	25.62
Interdigital necrobacillosis	140	0.38	51	25.1
Retained Placenta	93	0.25	37	18.2
Not recorded	81	0.22	45	22.2
Mastitis	41	0.11	28	13.8
Extraction/C-section	30	0.08	19	9.4
Gastro-intestinal[b]	30	0.08	21	10.3
Fever/depression/not doing well	25	0.07	17	8.4
Prolapse	26	0.07	20	9.9
Pneumonia	27	0.07	18	8.9
Abcess/cuts/cellulitis	20	0.05	15	7.4
Cancer eye	13	0.04	9	4.4
Lamenss	15	0.04	8	3.9
Non-antimicrobial treatments[c]	10	0.03	4	2.0
Lump-jaw/woody tongue	11	0.03	9	4.4
Eye or ear infection	9	0.02	8	3.9
Neurological	9	0.02	8	3.9
Infectious Keratoconjunctivitis	9	0.02	8	3.9
Kidney/bladder infection	2	0.01	2	1.0
Diarrhea	2	0.01	2	1.0
Coccidiosis	3	0.01	2	1.0
Vaginal tear	4	0.01	4	2.0
Prophylactic	5	0.01	4	2.0
Fetotomy	1	0.003	1	0.5
Dehorned	1	0.003	1	0.5
Ruminal tympany	1	0	1	0.5

[a] Individual animals may have had more than one diagnoses.
[b]Gastro-intestinal includes hardware and perotinits
[c]No antimicrobial treatments include treatments for lice, milk let down and milk fever

Table 3.6. Number (%) of herds recording various antimicrobial treatments used in cows/heifers from January 1 to June 30, 2002 (N=203)

Treatment	Never used Number (%)	Used 1-3 times Number (%)	Used 4-10 time Number (%)	Used >10 times Number (%)	Herds ever used
Cow LA Penicillin[a]	160 (78.8)	26 (12.8)	11 (5.4)	6 (3.0)	43 (21.2)
Cow SA Penicillin[b]	180 (88.7)	14 (6.9)	6 (3.0)	3 (1.5)	23 (11.4)
Cow oral sulphonamides	197 (97.0)	5 (2.5)	1 (0.5)	0 (0)	6 (3.0)
Cow trimethoprim/sulphsdiazine	192 (94.6)	10 (4.9)	0 (0)	1 (0.5)	11 (5.4)
Cow Oxytetrcycline LA	98 (48.3)	46 (22.7)	38 (18.7)	21 (10.3)	105 (51.7)
Cow Oxytetetrcycline SA	197 (97.0)	4 (2.0)	2 (1.0)	0 (0.0)	6 (3.0)
Cow tetracycline bolus	199 (98.0)	2 (1.0)	2 (1.0)	0 (0.0)	4 (2.0)
Cow tilmicosin	187 (92.1)	15 (7.4)	1 (0.5)	0 (0.0)	16 (7.9)
Cow florfenicol	194 (95.6)	8 (3.9)	1 (0.5)	0 (0.0)	9 (4.4)
Cow sulbactam-ampicillin injectable	197 (97.0)	1 (0.5)	0 (0.0)	5 (2.5)	6 (3.0)
Cow ceftiofur	200 (98.5)	3 (1.5)	0 (0.0)	0 (0.0)	3 (1.5)
Cow enrofloxacin tablets oral	203 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Cow novobiocin/ penicillin G ntramammary	199 (98.0)	2 (1.0)	1 (0.5)	1 (0.5)	4 (2.0)
Cow cephapirin sodium intramammary	200 (98.5)	2 (1.0)	0 (0.0)	1 (0.5)	3 (1.5)
Cow spectinomycin hydrochloride	202 (99.5)	1 (0.5)	0 (0.0)	0 (0.0)	1 (0.5)
Cow dihydrostreptomycin	202 (99.5)	1 (0.5)	0 (0.0)	0 (0.0)	1 (0.5)
Cow gentamicin injectable	202 (99.5)	0 (0.0)	1 (0.5)	0 (0.0)	1 (0.5)
Cow amprolium hydrochloride oral	202 (99.5)	1 (0.5)	0 (0.0)	0 (0.0)	1 (0.5)
Cow other	199 (98.0)	4 (2.0)	0 (0.0)	0 (0.0)	4 (2.0)
Cow unknown other	203 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)

[a]LA=Long acting
[b] SA=Short acting

Table 3.7. Number (%) of herds recording various antimicrobial treatments used in calves from January 1 to June 30, 2002. (N=203)

Treatment	Never used Number (%)	Used 1-3 times Number (%)	Used 4-10 times Number (%)	Used >10 times Number (%)	Herds ever used
Calf LA Penicillin[a]	189 (93.1)	7 (3.4)	1 (0.5)	6 (3.0)	14 (6.9)
Calf SA Penicillin[b]	177 (87.2)	9 (4.4)	11 (5.4)	6 (3.0)	26 (12.8)
Calf oral sulphonamides	100 (49.3)	24 (11.8)	35 (17.2)	44 (21.7)	103 (50.7)
Calf injectable sulphonamides	146 (71.9)	18 (8.9)	17 (8.4)	22 (10.8)	57 (28.1)
Calf Oxytetracycline LA	127 (62.6)	22 (10.8)	27 (13.3)	27 (13.3)	76 (37.4)
Calf Oxytetracyline SA	201 (99.0)	1 (0.5)	1 (0.5)	0 (0.0)	2 (1.0)
Calf tetracycline bolus	202 (99.5)	1 (0.5)	0 (0.0)	0 (0.0)	1 (0.5)
Calf tilmicosin	161 (79.3)	21 (10.3)	10 (4.9)	11 (5.4)	42 (20.6)
Calf florfenicol	119 (58.6)	20 (9.9)	33 (16.3)	31 (15.3)	84 (41.5)
Calf sulbactam-ampicillin injectable	187 (92.1)	8 (3.9)	1 (0.5)	7 (3.4)	16 (7.9)
Calf ceftiofur	187 (92.1)	4 (2.0)	6 (3.0)	6 (3.0)	16 (7.9)
Calf enrofloxacin tablets oral	202 (99.5)	1 (0.5)	0 (0.0)	0 (0.0)	1 (0.5)
Calf gentamicin injectable	201 (99.0)	2 (1.0)	0 (0.0)	0 (0.0)	2 (1.0)
Calf amprolium hydrochloride oral	199 (98.0)	1 (0.5)	2 (1.0)	1 (0.5)	4 (2.0)
Calf ampicillin trihydrate injectable	201 (99.0)	2 (1.0)	0 (0.0)	0 (0.0)	2 (1.0)
Calf erythromycin	202 (99.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Calf cephalexin oral	202 (99.5)	0 (0.0)	1 (0.5)	0 (0.0)	1 (0.5)
Calf other	193 (95.1)	10 (4.9)	0 (0.0)	0 (0.0)	10 (4.9)
Calf unknown other	198 (97.5)	2 (1.0)	1 (0.5)	2 (1.0)	5 (2.5)

[a]LA=Long acting
[b] SA=Short acting

Table 3.8. Number (%) of herds that used antimicrobials used at least once on the farm in the period from January 1 to June 30, 2002 (N=203)

Treatment	No use Number (%)	Used at least once Number (%)
Penicillin	126 (62.1)	77 (37.9)
Sulphonamide	83 (40.9)	120 (59.1)
Oxytetracycline/tetracycline	80 (39.4)	123 (60.6)
Tilmicosin	149 (73.4)	54 (26.6)
Florfenicol	115 (56.7)	88 (43.3)
Sulbactam-ampicillin injectable	183 (90.1)	20 (9.9)
Ceftiofur	185 (91.1)	18 (8.9)
Enrofloxacin	202 (99.5)	1 (0.5)
Intramammary products	197 (97.0)	6 (3.0)

Table 3.9. The herd-adjusted unconditional associations between non-therapeutic risk factors and the odds of calf treatment in 2002 (n=28,573, N=203)

Variable	Odds ratio	95% CI Lower	95% CI Upper	*P*-value
Vaccinated for BVDV/IBR prebreeding 2001[a]				
1. Live vaccine	2.6	0.5	13.1	0.71
2. Inactivated vaccine	1.6	0.3	8.1	0.95
3. No vaccine		Reference category		
4. No record	2.3	0.4	12.3	0.81
Heifers vaccinated for diarrhea (scours) precalving 2002	0.7	0.4	1.3	0.26
Cows vaccinated for diarrhea (scours) precalving 2002	0.7	0.4	1.3	0.32
BCS at pregnancy testing (9-point scale) [a]				
1. BCS <5 compared to BCS ≥5	0.9	0.8	1.1	0.77
2. Missing BCS compared to BCS ≥5	1.4	1.2	1.7	0.0001
Cow purchased	1.2	1.0	1.3	0.03
Predominant breed type[a]				
1. British		Reference category		
2. Continental	1.0	0.9	1.2	0.99
3. Cross	0.8	0.6	1.1	0.73
4. No record	1.1	0.7	1.8	0.96
Age category [a]				
1. Yearling heifer	0.8	0.2	2.9	0.99
2. 2 year old heifer (born 1999)	1.2	1.1	1.4	0.08
3. 3 year old cow (born 1998)	1.1	1.0	1.3	0.51
4. Mature cow (born 1992 to 1997)		Reference category		
5. Cow age >10 (born in 1991 or earlier)	0.9	0.8	1.1	0.91
6. No record of age	1.2	0.9	1.8	0.93
Age re-categorization[a]				
Yearling heifer	0.7	0.2	2.7	0.62
All other breeding females		Reference category		
Problem reported with cow at calving[a]				
1. Nothing		Reference category		
2. Prolapse	1.5	0.6	3.6	0.84
3. Retained fetal membranes	1.4	1.0	1.9	0.43
4. Metritis	2.0	0.2	20.6	0.95
Calving assistance reported[a]				
1. No assistance		Reference category		
2. Malpresentation and/or Pull	1.6	1.4	1.8	0.0001
3. Caesarian section surgery	2.2	1.3	3.7	0.012
Calf sex[a]				
1. Male		Reference category		
2. Female	0.8	0.7	0.8	0.0001
3. Unknown	0.4	0.2	0.6	0.0001
Precipitation growing season 2001[a]				
1. 75 to 200 mm	2.2	1.1	4.3	0.09
2. 200 to 250 mm	2.1	1.1	4.4	0.11
3. >250 mm		Reference category		
% Cows ever treated in the herd	1.2	1.2	1.3	0.003

[a]*P*-value based on degrees of freedom determined by the number of levels of the categorical variable

Table 3.10. The herd-adjusted final multivariable analysis of risk factors for whether a calf was ever treated between January and June, 2002 (n=28,573, N=203)

Variable	Odds ratio	95% CI		*P*-value
		Lower	Upper	
Calf gender				
1. Male		Reference category		
2. Female	0.8	0.7	0.9	0.0001
3. Not recorded	0.4	0.3	0.6	0.0006
Calving assistance reported				
1. No assistance		Reference category		
2. Malpresentation and/or pull	1.5	1.3	1.7	0.0001
3. Caesarian section surgery	2.0	1.2	3.3	0.15
% of cows treated in the herd[a]	1.2	1.1	1.3	0.002

[a]The percentage of cows treated in a herd was calculated from the number of treatments administered to any adult female in the herd between January 1 and June 30, 2002 and the total number of adult females in that herd as of January 1, 2002.

Table 3.11. The herd-adjusted unconditional associations between non-therapeutic risk factors and the odds of cow/bred heifer treatment in 2002 (n=31,248, N=203)

Variable	Odds ratio	95% CI Lower	95% CI Upper	*P*-value
Predominant breed type[a]				
1. British		Reference category		
2. Continental	0.9	0.7	1.3	0.98
3. Cross	0.4	0.3	0.8	0.03
4. No record	0.3	0.1	1.2	0.42
Age category[a]				
1. 2 year old heifer (born 2000)	1.3	1.0	1.6	0.25
2. 3 year old cow (born 1999)	1.0	0.8	1.3	0.99
3. Mature cow (born 1993 to 1998)		Reference category		
4. Cow age >10 (born in 1992 or earlier)	1.2	1.0	1.6	0.57
5. No record of age	0.6	0.2	1.6	0.96
Vaccinated for BVD/IBR prebreeding[a]				
1. Not Vaccinated	1.8	1.1	2.3	0.07
2. Vaccination status not reported	0.3	0.1	1.6	0.41
3. Vaccinated		Reference category		
Vaccinated for diarrhea (scours) precalving[a]				
1. No breeding females vaccinated	2.0	1.2	3.2	0.006
2. Breeding females vaccinated		Reference category		
Calving assistance reported[a]				
1. No assistance		Reference category		
2. Presentation correction or pull	2.8	2.2	3.4	0.00001
3. C-section	13.2	8.3	21.0	0.00001
Problem reported with cow at calving[a]				
1. No problem		Reference category		
2. Prolapse	68.5	35.9	130.9	0.00001
3. Retained fetal membranes	114.9	82.3	160.3	0.00001
4. Metritis	304.6	47.4	1956.7	0.00001

[a]*P*-value based on degrees of freedom determined by the number of levels of the categorical variable

Table 3.12. The herd-adjusted final multivariable analysis of risk factors for whether cows and bred heifers were ever treated between January and June, 2002 (n=31,248, N=203)

Variable	Odds ratio	95% CI		*P*-value
		Lower	Upper	
Calving assistance reported				
1. No assistance		Reference category		
2. Malpresentation and/or Pull	2.1	1.7	2.7	0.0001
4. Caesarian section surgery	14.5	8.8	23.8	0.0001
Problem reported with cow at calving				
1. Nothing		Reference category		
2. Prolapse	56.5	29.0	110.0	0.0001
3. Retained fetal membranes	109.2	77.9	153.0	0.0001
4. Metritis	311.7	50.1	1940.5	0.0001

CHAPTER 4
PREVALENCE OF ANTIMICROBIAL RESISTANCE IN FECAL GENERIC *E. COLI* ISOLATED IN WESTERN CANADIAN COW-CALF HERDS. PART I: BEEF CALVES

4.1. Introduction

The emergence of antimicrobial resistance (AMR) is a serious concern in both human and veterinary medicine. Antimicrobial resistant bacteria were first observed shortly after the discovery of penicillin (North and Christie, 1946, Barber, 1947), and resistance has continued to surface with the introduction of each new antimicrobial compound (Levy, 1997). It is generally accepted that antimicrobial use (AMU) is an important factor for the selection of AMR bacteria (Aarestrup, 1999, van den Bogaard and Stobberingh, 2000, McEwen and Fedorka-Cray, 2002). Selective pressure for AMR can be affected by treatment formulation, dose, interval, and duration (Catry et al., 2003). Because AMU varies widely within the livestock industry, describing AMR in one livestock class, species, or management system will not be representative of other systems.

Most AMU and AMR research in the food animal sector has been conducted in swine, poultry or feedlot operations (Dargatz et al., 2003, Fitzgerald et al., 2003, Lanz et al., 2003, Bywater et al., 2004, Khachatryan et al., 2004, Rajic et al., 2004, Hershberger et al., 2005). Animals in intensively managed facilities can be exposed to

antimicrobials in feed, in water, or via metaphylaxis protocols involving injectable formulations (McEwen and Fedorka-Cray, 2002). Feed antimicrobials are uncommon in cow-calf herds, and injectable AMU is infrequent especially in adult animals (Gow and Waldner, 2007). The selective pressures experienced in cow-calf herds may differ and, therefore, lead to fewer AMR bacteria compared to other food animal populations that are exposed to more intensive selective pressure associated with routine AMU practices.

Although there is a growing amount of literature on AMR, to the best of our knowledge, there is no information currently available regarding AMR in cow-calf herds in western Canada. The cow-calf industry is a vital and important part of the agricultural economy in all parts of Canada, but particularly in Saskatchewan and Alberta. These two provinces are home to more than 65% of the beef cow, breeding heifer, and calf populations in Canada (Statistics Canada, Accessed July 25, 2006; http://www.40.statcan.ca/101/cst01/prim50a.htm). A better understanding of AMR in this industry is essential to develop baseline data and determine the need for future monitoring. The objective of this study was to describe AMR patterns in calves from western Canadian cow-calf herds in the spring and fall of 2002 using *E. coli* as an indicator organism.

4.2. Materials and methods

4.2.1. Study overview

This project was one step in a larger initiative to examine the prevalence of and risk factors associated with AMR in cow-calf herds (Figure 4.1). Targeted sampling was initiated in 2002 to investigate the prevalence of AMR at different stages of production. Fecal samples were collected in the spring from cows and calves. These samples were not from cow-calf pairs, and not all of the same herds had both cow and calf samples collected. Calves were also sampled in the fall near the time of weaning. Due to logistical constraints, not all of the same herds and none of the same calves were sampled in both time periods. This analysis focuses on the calves sampled in the spring and fall of 2002. In part II of this study, the prevalence of AMR is described for the cows sampled in the spring of 2002 and for cow-calf pairs sampled in the spring of 2003 (Gow et al., 2007).

4.2.2. Background and herd selection

Fecal samples were collected from a convenience subset of herds participating in a survey of risk factors affecting cattle productivity and health (https://www.wissa.info). Private veterinary clinics across Alberta, Saskatchewan, and north-eastern British Columbia were approached and asked to participate. Within each practice, herds were identified and enrolled based on the selection criteria which considered factors such as herd size (>50 cows), animal identification, existing calving records, animal handling facilities sufficient for pregnancy testing and bull evaluation, and a relationship with a local veterinary clinic. Participating herds were visited at least quarterly by one of six

veterinarians hired to collect data and samples. Fecal samples for this AMR study were collected between January and May 2002 for the calves sampled in the spring, and between September and December 2002 for the calves sampled in the fall.

4.2.3. Sample collection

Fecal samples were collected in the spring from 480 individually identified calves that were accessible in the calving and nursery area on 91 privately owned cow-calf farms in Alberta and Saskatchewan. Fecal samples were also collected from 394 calves on 45 farms while calves were being handled for fall processing procedures such as vaccination, castration, and sorting for sale. All fecal samples were obtained either directly from the rectum or from the ground immediately after defecation. A separate disposable glove and container were used for each animal sampled.

4.2.4. Laboratory methods

4.2.4.1. *Escherichia coli* culture

Fecal samples were sent on ice to a diagnostic laboratory (Prairie Diagnostic Services, Saskatoon, Saskatchewan) for culture. The samples were cultured onto MacConkey agar plates at 37°C for 18 hours for isolation of *E. coli*. At least three individual lactose fermenting colonies identified as *E. coli* using standard biochemical tests, including indole, triple sugar iron (TSI) slant, citrate, and urea, were saved from each sample. If both dry and mucoid colonies were detected within a sample, then three

isolates from each colony type were tested. Individual *E. coli* isolates were stored in 50% glycerol and Luria-Bertani (LB) broth at -80ºC.

4.2.4.2. Susceptibility testing methodology

E. coli isolates were tested for susceptibility (Alberta Agriculture and Food) using microbroth dilution (Sensititre®, TREK Diagnostic Systems Inc., Cleveland, Ohio) and the standard 2002 National Antimicrobial Resistance Monitoring System (NARMS) CMV7CNCD gram negative public health panel (CIPARS, 2006).

Minimum inhibitory concentrations (MICs) were assessed for sixteen antimicrobial agents (Table 4.1). Breakpoints for susceptibility were used as defined by the National Committee of Clinical Laboratory Standards (NCCLS) (NCCLS, 2000). All isolates in the intermediate susceptibility range were classified as susceptible. Amikacin results > 4µg/mL were labeled not interpretable because the breakpoint is 4 dilutions beyond the range of the panel. The breakpoint used for streptomycin was 64µg/ml (CIPARS, 2006).

For the antimicrobials tested the minimum inhibitory concentration were presented classified according to the Veterinary Drug Directorate (VDD), Health Canada Guidelines (Figure 2) (CIPARS, 2006). Category I antimicrobials are considered to have very high importance in human medicine and from the gram negative NARMS 2002 public health panel include ceftiofur, ceftriaxone, and ciprofloxacin. Category II includes drugs considered highly important in human medicine such as: amikacin, amoxicillin/clavulanic acid, gentamicin, kanamycin, nalidixic acid, streptomycin, and

trimethoprim/sulphamethoxazole. Category III antimicrobials are of medium importance and include: ampicillin, cefoxitin, cephalothin, chloramphenicol, sulphamethoxazole, and tetracycline. To facilitate consistent comparisons with the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) (http://www.phac-aspc.gc.ca/cipars-picra), the same nomenclature for patterns of resistance were used (CIPARS, 2006). Multiple resistance was defined as resistance to ≥2 antimicrobials.

4.2.5. Statistical analysis

Descriptive analyses were completed using a commercially available software program (SPSS 11.0 for Windows, SPSS Inc., Chicago, Illinois). If any one isolate from a calf was resistant to a particular antimicrobial, then that calf was identified as being positive for resistance to that antimicrobial. Additionally, if any calf from a herd was classified as positive, then the herd was also reported as positive for resistance to that antimicrobial.

Population-average prevalence estimates and 95% confidence intervals (CI) for AMR were determined using the intercept from null models. Models were developed using generalized estimating equations (GEE) to account for clustering within herd (SAS v.8.2 for Windows (PROC GENMOD); SAS Institute, Cary, North Carolina, USA). Model specifications included a binomial distribution, logit link function, repeated statement with subject equal to herd, and an exchangeable correlation

structure. Where the proportion of isolates with resistance was equal to zero, Fleiss quadratic 95% CI formulas for a single proportion were calculated (Fleiss et al., 2003).

Using the same model specifications described above, unconditional associations between resistance to any antimicrobial and calf age (categorized by quartiles), calf gender, calf breed, whether the calf was ever treated prior to sampling, and the number of days since the last treatment were investigated separately for the spring and fall samples (treated calves only). In addition to the above predictors, dam age (2 years, 3 years, 4 to 10 years, and >10 years) was also considered when modeling calf AMR status in the spring.

For the twenty herds sampled in both the spring and the fall of 2002, we investigated whether the proportion of isolates or calves with resistance in the spring was a predictor of the proportion (count of AMR positive isolates (or calves) / number of isolates (or calves) collected) with resistance in the fall using GEE and the above model specifications.

The frequency of AMR was also compared between the spring and fall where both sets of samples were available for the same herd using GEE with the model specifications outlined above. The total number of calves with any type of AMR (numerator) as a proportion of the total number of calves sampled (denominator) was compared between when the herds were tested in the spring and fall.

The extent of clustering of isolate resistance within individual calves and herds was described for the samples collected in the spring. The variance components for a three-level model were estimated using penalized quasi-likelihood estimates (2nd order PQL) (MLwiN version 2.0, Centre for Multilevel Modeling, Institute of Education, London, UK), a binomial distribution, and logit link function. Data from this null model were used to estimate the variation at the isolate level (n=1677) ($\rho_i=\pi^2/3/ (\sigma^2_h+ \sigma^2_c +\pi^2/3)$), calf level ($\rho_c= \sigma^2_c/(\sigma^2_h+ \sigma^2_c +\pi^2/3)$), and herd level ($\rho_h= \sigma^2_h/(\sigma^2_h+ \sigma^2_c +\pi^2/3)$) (Dohoo et al., 2003). The low prevalence of AMR in the samples collected in the fall and the lack of variation only allowed for variation estimates to be calculated for a two-level model; if a third level for calf was included the model would not converge. The proportion of variation was reported for isolates (n=1187) within herds ($\rho=\pi^2/3(\sigma^2_h +\pi^2/3)$) and between herds ($\rho=\sigma^2_h/(\sigma^2_h +\pi^2/3)$) (Dohoo et al., 2003).

4.3. Results

4.3.1. Study conducted in the spring of 2002

From the 480 calves sampled (212 female and 268 male), 1677 isolates were recovered for further testing. Healthy calves made up 92.5% (444/480) of the sample population. Calf age ranged from 0 to 151 days (median, 6; inter-quartile range (IQR), 4 to 10). Median herd size (N=91) was 177 (range, 89 to 411) breeding females. The median number of samples collected per herd was 5 (range, 1 to11; IQR, 4 to 6). Before sample collection, 8.3% (40/480) of calves had been reported as treated with either oral

or injectable antimicrobials or both. Calf age at last treatment prior to sample collection ranged from 0 to 56 days of age (median, 2; IQR, 0 to 7). For treated calves, the number of days between last treatment and sample collection ranged from 0 to117 days (median, 5; IQR, 1 to 10).

4.3.2. Observed AMR in the calves sampled in the spring of 2002

Resistance to at least one antimicrobial was identified in 48.8% of isolates, 62.2% of calves, and in 91% of herds (Tables 4.1 to 4.3). The two antimicrobials to which resistance was most commonly identified were tetracycline and sulphamethoxazole. No resistance was identified to ceftriaxone and ciprofloxacin, and low levels of resistance were identified for ceftiofur and gentamicin.

The maximum number of antimicrobials to which an isolate demonstrated resistance was 10. Resistance to at least 6 antimicrobials was observed in 9.4% (157/1677) of the isolates; these highly resistant isolates were identified in 10.2% (49/480) of calves and 14% (13/91) of herds. The most common pattern found in the multiresistant isolates was ampicillin, kanamycin, streptomycin, sulphamethoxazole, tetracycline, and trimethoprim/sulphamethoxazole. Of the isolates with resistance to at least six antimicrobials, 75.8% (119/157) had a pattern including streptomycin, sulphamethoxazole, tetracycline, and trimethoprim/sulphamethoxazole resistance.

No resistance was detected to the Category I antimicrobials except for one isolate that had resistance to ceftiofur (Figure 4.2). The median MIC ranges for Category I, II,

and III antimicrobials were several dilutions away from the break point, except for streptomycin and tetracycline respectively.

Calf AMR status was not significantly associated with calf gender (P=0.54), breed (P=0.40), dam age (P=0.72), or if the calf was ever treated (P=0.65) prior to sample collection. Calves 0 to 3 days of age were 0.55 (95% CI, 0.30 to 1.0; P=0.04) times as likely to be positive for any AMR as calves >10 days of age. The AMR status of calves 4 to 5 and 6 to 9 days of age were not significantly different from calves ≥10 days of age (P>0.49). For the calves that had been treated, the number of days from last treatment was not associated with the presence of AMR (P=0.92).

In the null model the proportion of variance in AMR accounted for at the isolate, calf, and herd levels was 65.1%, 14.6%, and 20.3%.

4.3.3. Study conducted in the fall of 2002

Samples were collected from 394 healthy calves (242 female, 152 male) on 45 farms. Calf age ranged from 118 to 323 days (median, 219); 79% of the samples were collected from calves less than 250 days of age. The median number of samples collected per herd was 10 (range, 1 to10; IQR, 10 to 10), and the median herd size was 125 (range, 52 to 265) breeding females. Before sample collection, 10.6% (39/367) of the calves had been treated with either an oral or injectable antimicrobial or an oral and injectable antimicrobial together. Calf age at last treatment ranged from 0 to 46 days of age (median, 14 days; IQR, 10 to 20). For treated calves the median number of days

between sample collection and the calf's last treatment was 186 days (range, 140 to 284; IQR, 178 to 208).

4.3.4. Observed AMR in the calves sampled in the fall of 2002

AMR was relatively less common in the 1186 isolates recovered from the fall samples; 7.0% were resistant to at least one antimicrobial (Table 4.1). At least one resistant isolate was identified in 12.7% of calves and 56% of herds (Table 4.2 to 4.3). The majority of the resistance detected was to tetracycline and sulphamethoxazole. No resistance was identified for several antimicrobials including: ceftiofur, ceftriaxone, cefoxitin, ciprofloxacin, and gentamicin.

The maximum number of antimicrobials to which an isolate demonstrated resistance was 5; 0.4% of isolates and 0.8% of calves demonstrated resistance to 5 antimicrobials. The most common pattern found in multiresistant isolates was streptomycin, sulphamethoxazole, and tetracycline.

No resistance was detected to the Category I antimicrobials, and the median MIC ranges for these antimicrobials were several dilutions away from the break point (Figure 4.3).All of the median MICs for the Category II and III antimicrobials were also several dilutions below the breakpoint. The exceptions were streptomycin, which was immediately below the breakpoint (Figure 4.3), and tetracycline and cephalothin, which were only two dilutions below the breakpoint (Figure 4.3).

Calf AMR status in the fall was not associated with calf age (P=0.75), gender (P=0.85), breed (P=0.38), and whether the calf had ever been treated (P=0.13) prior to sample collection. For the calves that had been previously treated, the number of days since last treatment (P=0.74) was not associated with any AMR. Based on a two-level model of these data, 84.9% of the total variation in AMR was accounted for between isolates within herds and 15.1% was accounted for by variation between herds.

4.3.5. Association between the prevalence of resistance in calf samples collected in the spring and the occurrence of resistance in calves in the fall

In the 20 herds sampled at both time points, the proportion of isolates and calves that were positive for resistance in the spring were not statistically significant predictors of the proportion of isolates (P=0.82) or calves positive (P=0.37) for resistance in the fall. Beef calves sampled in the spring were 9.6 (95% CI, 4.5 to 20.7) times more likely to have at least one resistant isolate than those sampled in the fall from the same herds.

4.4. Discussion

Information is needed to determine the extent and severity of AMR in the cow-calf industry given that these are the most common livestock operations in western Canada and that veterinary supervised herd health programs in these herds are still relatively uncommon compared to other commodities. This study, which provides some of the first available on-farm data describing AMR in cow-calf herds, found that resistance to antimicrobials identified as very important in human medicine was rare. *E. coli* isolates

from both the spring and fall samples were most commonly resistant to tetracycline, sulphamethoxazole, and streptomycin. This finding is consistent with other reports of AMR in *E. coli* isolates collected from a variety of different animal species (Kijima-Tanaka et al., 2003, Bywater et al., 2004, Khachatryan et al., 2004). The other key finding of this study was that young calves sampled in the spring had a higher prevalence of AMR than older calves sampled in the fall.

While it is difficult to directly compare AMR across food animal studies due to methodological differences, general trends have been noted. Even though the most common resistances detected are relatively similar between livestock species and management systems, the proportion of resistant organisms vary. For example, broilers tended to have more resistant *E. coli* isolates than healthy swine or beef cattle (Kijima-Tanaka et al., 2003). Tetracycline resistance was detected in 69% of the broiler isolates and in 25 % of the cattle isolates (Kijima-Tanaka et al., 2003). Schroeder et al. (2002) demonstrated that swine carried the highest number of resistant isolates when compared to human, cattle, and food diagnostic samples. Resistance to sulphamethoxazole and tetracycline was detected in 74% and 71% of the swine isolates but in only 14% and 20% of cattle isolates (Schroeder et al., 2002). Because the prevalence of AMR varies across species, studying resistance in one livestock species is not necessarily representative. In order to appreciate the range of AMR and the effect of AMU practices in agriculture, industry specific investigations, as reported here, are required.

Resistance to antimicrobials that were classified as very important in human medicine was detected in less than 1% of the isolates in this study. Additionally, for the majority of antimicrobials of interest to human medicine, the median MICs were well below the breakpoint for resistance. Median MICs several dilutions below the breakpoint indicate that the *E. coli* populations in these calves were highly sensitive to those antimicrobials. Based on these findings, it appears that on-farm exposure to beef calves presents a low risk to human health. Hoyle et al. (2004) reported much higher levels of ampicillin (64%) and nalidixic acid (24%) resistance in calves on a Scottish beef farm. The discrepancy in prevalence may be the result of a variation in selection pressure due to different management systems.

Chloramphenicol resistance was detected despite the ban of chloramphenicol use in Canadian food producing animals since 1985 (Gilmore, 1986). This may indicate that co-selection was maintaining chloramphenicol resistance genes in the population. The persistence of chloramphenicol resistance despite the elimination of chloramphenicol use has also been reported by national surveillance programs in Japan and in Canada (Kijima-Tanaka et al., 2003, CIPARS, 2006). Molecular studies are needed to further examine this question in cow-calf herds.

Individual calf attributes such as dam age, calf gender, breed, and whether the calf had ever been treated prior to sampling were not associated with the occurrence of resistance in the beef calves in this study. However, resistance was less common in calves less than 3 days of age than in calves that were at least 10 days of age. This

finding may be due to a greater opportunity for colonization with resistant organisms, gained either from the environment or other animals in the herd, with increasing calf age. A similar result was previously reported by Berge et al. (2005a) who described a higher level of AMR in 2 week old dairy calves as compared to day old calves.

In the current study, there was no association between the number of days from last treatment and the presence of AMR in treated calves. Berge et al. (2005a) indicated that the effect of individual animal treatment was transitory and was associated with AMR if the sample was collected less than 7 days post treatment. This discrepancy could be the result of differences in selection pressures between the two groups or the result of host specific differences between beef and dairy calves. It may also be due to different approaches in methodology and in statistical analyses between the two projects. Berge et al. (2005a) examined dairy calves longitudinally, they allocated isolates to AMR clusters based on mean zone size diameters, and Monte Carlo simulation of the non-parametric Jonckeheere-Terpstra test was used to examine days from last treatment and cluster membership. The current project used a single sample collection for each animal, AMR was considered present or absent, and GEE logistic regression was used to examine the relationship between time from treatment and AMR.

In this study, herds with a high prevalence of AMR in the spring were not more likely to have a high prevalence in the fall. These findings suggest that AMR profiles are not static and may be affected by a number of factors potentially including calf physiology and environment. The transitory nature of AMR has also been demonstrated

in the feedlot. In the feedlot AMR levels shifted towards a uniform population dictated by the feedlot environment regardless of AMR prevalence at arrival (Berge et al., 2005b).

Beef calves sampled in the spring were almost ten times more likely to shed resistant organisms than those sampled in the fall. The reason for this difference is unknown. There was no association in these data between individual calf treatment history and the occurrence of resistance. Other factors that might explain this finding include an increased intensity of herd AMU in the spring calving season compared to the summer pasture season, an increased degree of crowding and opportunity for AMR transmission in the spring calving season compared to the summer pasture season, and the status of the dam at calving were not addressed directly in this part of the study.

The age-related differences in calf physiology between the first few weeks of life and weaning might also explain the difference in AMR prevalence. Young calves are pre-ruminants, are on a milk based diet, and are primarily housed in close confinement. Older calves in the fall are ruminants, on a forage based diet, and are usually managed extensively on pasture before weaning. This study was not specifically designed to examine the associations between calf age and the difference in AMR between the spring and fall samples. To assess this association, individual calves could be followed longitudinally from birth through to weaning. Any changes in AMR prevalence with age could then be detected and potentially differentiated from the influence of herd level AMU and other management practices.

Previous research has demonstrated that calves rapidly acquire AMR bacteria within days of birth (Hoyle et al., 2004). The presence of AMR in these animals is not necessarily related to AMU (Khachatryn et al., 2004, Berge et al.; 2005a), but rather animal age (Hinton et al., 1984, Hinton, 1985, Brophy et al., 1977, Mathew et al., 1999, Khachatryn et al., 2004). Typically AMR is highest in young animals (Khachatryn et al., 2004) and declines linearly with age (Hoyle et al., 2004). This phenomenon has not previously been described in beef calves and is not fully understood in other species and production environments

One possibility is that the decline in AMR could be an artifact reflecting no change in the proportion of resistant organisms, but rather the overall decline in total *E. coli* with the absolute number of resistant bacteria falling below the detection limits of the test in older animals (Hoyle et al., 2004). Although a natural gradual reduction of *E. coli* with increasing age has been previously been reported (Smith and Crabb, 1961), the decline in *E. coli* as an animal matures does not appear to explain the decrease in the AMR organisms detected.

Hoyle et al. (2004) demonstrated that beef calves preferentially lost resistant relative to susceptible bacteria as they aged. Additionally, other research has indicated that, in the absence of antimicrobials, AMR could be maintained because SSuT strains had a fitness advantage in young calves but not in older animals (Khachatryan et al., 2004). The presence of these resistant *E. coli* in the absence of treatment and selective pressure

could be due to fitness traits that make them better able to compete in the calf gut compared to susceptible organisms. These traits may include non-scavenging mechanisms (siderophores), increased adhesion, and mechanisms that enhance colonization, reproduction, and spread (Visca et al., 1991, Allen et al., 1993, Simmons et al., 1988, Mandal et al., 2001).

Because there are still many unknowns regarding the determinants of resistance in these herds, a multi-level analysis was used to attempt to determine where most of the unexplained variation exists in the occurrence of AMR and potentially where interventions could be most successfully targeted. The majority of variation detected was at the isolate level. Since interventions cannot be applied to the isolate, potential AMR risk factors and AMR reducing interventions should be investigated at the calf and then herd level. However, in this study no specific individual animal risk factors were identified suggesting the need to look further at the calf's environment within the herd.

This is the first available information describing the prevalence of AMR in calves from western Canadian beef herds during the calving season and at weaning. Baseline information is necessary to measure variation resulting from changing production practices and to develop strategies to control AMR emergence. Knowledge of stage of production and timing of sample collection is critical to interpreting surveillance data from these herds. Additional research is needed to understand why AMR varies between the groups targeted in this study. Future studies should consider animal age,

season, AMU, and herd management. Continued monitoring of AMR patterns in cow-calf herds will illustrate any emerging issues potentially important to public health.

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Figure 4.1. Sampling structure for study of AMR in western Canadian cow-calf herds

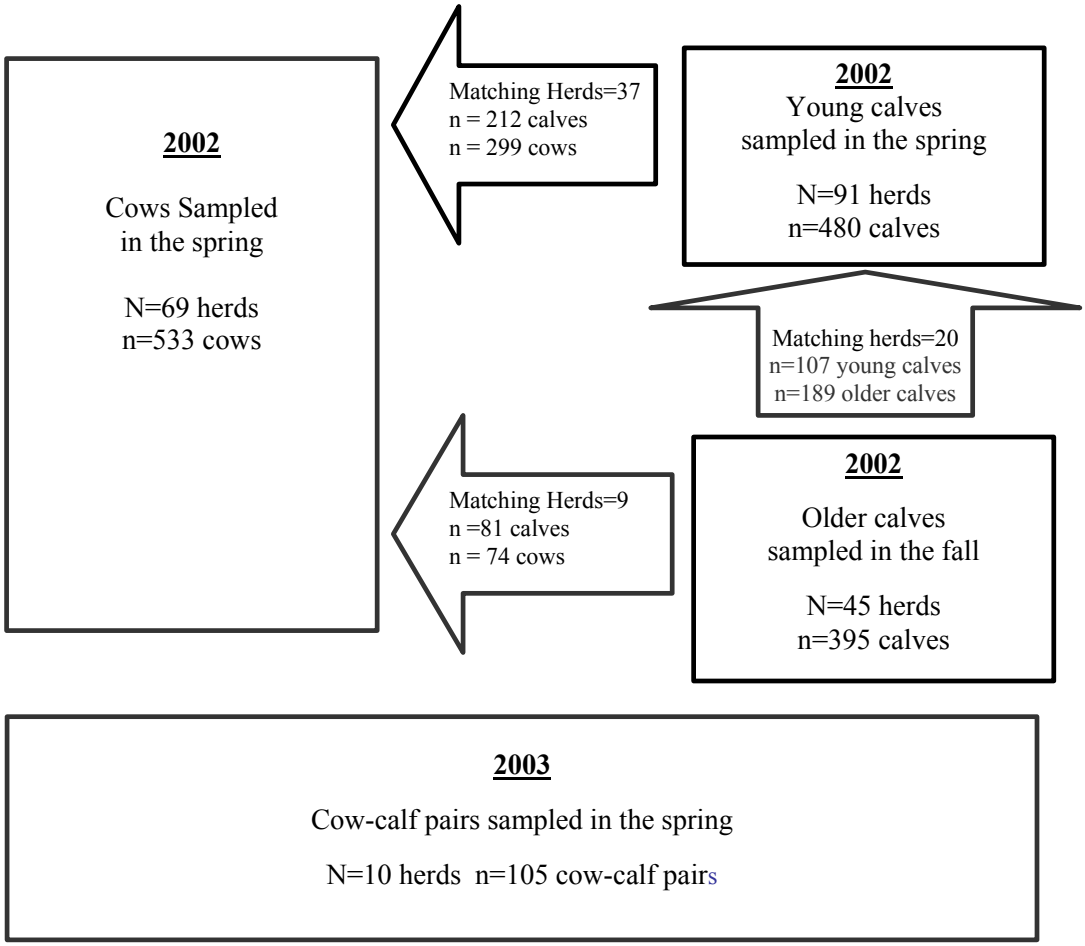

Figure 4.2. Minimum inhibitory concentrations for fecal *E. coli* isolates collected from calves in the spring of 2002 arranged by the Veterinary Drug Directorate, Health Canada, classification of drugs and presented as a percentage of the total number of isolates (N=1677)

*	Antimicrobial	n	MIC Percentiles		Distribution (%) of MICs																
			Median	75th	<=0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	>512
I	Ceftiofur	**1677**	**0.25**	**0.25**				3.2	72.5	20.1	1.8	0.1	0.8	0.9	**0.6**						
	Ceftriaxone	**1677**	**<=0.25**	**0.25**					95.8	1.8	0.2	0.2	0.8	0.8	0.5	0.1					
	Ciprofloxacin	**1677**	**<=0.015**	**<=0.015**	99.4	0.4	0.1	0.2													
II	Amikacin	**1677**	**2**	**2**						0.5	29.6	65.1	4.4	**0.4**							
	Amoxicillin-Clavulanic Acid	**1677**	**4**	**8**							2.7	22.0	47.6	16.9	6.1	2.1	**2.6**				
	Gentamicin	**1677**	**1**	**1**					10.0	24.9	63.7	0.6		0.3	0.4	**0.2**					
	Kanamycin	**1677**	**<=8**	**<=8**										77.1	0.1			**22.8**			
	Nalidixic Acid	**1677**	**4**	**4**							0.8	30.6	65.9	2.5	0.1		**0.2**				
	Streptomycin	**1677**	**<=32**	**64**												62.6	20.9	**16.5**			
	Trimethoprim-Sulphamethoxazole	**1677**	**<=0.12**	**0.5**				52.6	14.7	12.0	0.9			**19.8**							
III	Ampicillin	**1677**	**4**	**>=64**							2.8	34.9	33.3	2.8	0.9	0.2	**25.1**				
	Cefoxitin	**1677**	**4**	**4**							0.2	20.6	56.0	17.4	1.6	**4.2**					
	Cephalothin	**1677**	**8**	**16**								1.0	13.4	57.5	21.0	1.6	**5.5**				
	Chloramphenicol	**1677**	**8**	**8**								2.1	39.6	39.1	1.4	0.2	**17.5**				
	Sulphamethoxazole	**1677**	**<=16**	**>512**											52.9	0.1		0.1		0.4	**46.6**
	Tetracycline	**1677**	**8**	**>=64**									49.1	1.1	0.2	1.6	**48.0**				
IV																					

Note: Roman numerals I-III indicate the ranking of human importance, established by the Veterinary Drug Directorate, Health Canada. The unshaded fields indicate the range tested for each antimicrobial in the plate configuration. Vertical double bars indicate the breakpoints and highlighted cells locate the median. Numbers in bold font are the number of isolates with growth in all wells within the tested range, indicating the actual MIC is greater than that range of dilutions.

Figure 4.3. Minimum inhibitory concentrations for fecal *E. coli* isolates collected from calves in the fall of 2002 arranged by the Veterinary Drug Directorate, Health Canada, classification of drugs and presented as a percentage of the total number of isolates (N=1186)

*	Antimicrobial	n	MIC Percentiles		Distribution of Isolates (%) Across Minimum Inhibitory Concentrations (MIC) Ranges															
			Median	75th	<=0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	>512
I	Ceftiofur	1186	0.25	0.25				5.2	76.1	18.6											
	Ceftriaxone	1186	<=0.25	0.25					99.9	0.1											
	Ciprofloxacin	1186	<=0.015	<=0.015	99.7	0.3															
II	Amikacin	1186	2	2						3.4	42.3	52.9	1.3	**0.2**							
	Amoxicillin-Clavulanic Acid	1186	4	4							1.4	24.5	63.5	10.2	0.4						
	Gentamicin	1186	1	1					21.1	26.5	52.3	0.2									
	Kanamycin	1186	<=8	<=8										98.9				**1.1**			
	Nalidixic Acid	1186	4	4						0.1	1.3	43.7	53.7	1.3							
	Streptomycin	1186	<=32	<=32												97.2	2.1	**0.7**			
	Trimethoprim-Sulphamethoxazole	1186	<=0.12	<=0.12				94.2	4.9	0.7				**0.3**							
III	Ampicillin	1186	4	4							5.0	39.0	50.8	3.3	0.3	0.3	**1.3**				
	Cefoxitin	1186	4	4						0.1	0.1	25.3	62.3	11.7	0.5						
	Cephalothin	1186	8	8								0.9	15.7	66.4	16.4	0.5	**0.1**				
	Chloramphenicol	1186	4	8								7.9	59.0	31.6	0.8		**0.6**				
	Sulphamethoxazole	1186	<=16	<=16											96.0						**4.0**
	Tetracycline	1186	<=4	<=4									92.6	2.4	0.4	0.1	**4.6**				

Note: Roman numerals I-III indicate the ranking of human importance, established by the Veterinary Drug Directorate, Health Canada. The unshaded fields indicate the range tested for each antimicrobial in the plate configuration. Vertical double bars indicate the breakpoints and highlighted cells locate the median. Numbers in bold font are the number of isolates with growth in all wells within the tested range, indicating the actual MIC is greater than that range of dilutions.

Table 4.1. Prevalence (%) of AMR for *E. coli* isolates cultured from calves in the spring (n=1677) and in the fall (n=1186) of 2002 adjusted for clustering by herd

	Isolate prevalence for calves in the spring			Isolate prevalence for calves in the fall		
Antimicrobial	Prevalence (%)	Lower CI	Upper CI	Prevalence (%)	Lower CI	Upper CI
Amikacin	0.0	0.0	0.3	0.0	0.0	0.4
Amoxicillin-Clavulanic Acid	4.5	2.6	7.8	0.0	0.0	0.4
Ampicillin	22.7	18.0	28.2	1.6	0.7	3.3
Cefoxtin	4.1	2.3	7.2	0.0	0.0	0.4
Ceftiofur	1.7	0.7	3.8	0.0	0.0	0.4
Ceftriaxone	0.0	0.0	0.3	0.0	0.0	0.4
Cephalothin	6.7	4.3	10.1	0.6	0.3	1.4
Chloramphenicol	14.8	10.8	19.9	0.6	0.2	1.8
Ciprofloxacin	0.0	0.0	0.3	0.0	0.0	0.4
Gentamicin	0.5	0.2	1.3	0.0	0.0	0.4
Kanamycin	20.7	16.1	26.2	1.1	0.4	2.8
Nalidixic Acid	0.2	0.02	1.2	0.0	0.0	0.4
Streptomycin	34.8	29.4	40.7	2.8	1.6	4.9
Sulphamethoxazole	42.8	36.9	48.9	4.0	2.7	6.1
Tetracycline	46.4	40.2	52.7	5.0	3.4	7.5
Trimethoprim-Sulphamethoxazole	16.3	12.2	21.4	0.3	0.1	1.0
AMR (≥1 antimicrobial)	48.8	42.6	55.1	7.0	4.8	9.9
Multi AMR (≥2 antimicrobials)	46.2	40.1	52.5	5.5	3.7	8.2
A3C[a]	1.6	0.7	3.8	0.0	0.0	0.4
ACSSuT[b]	2.6	1.3	5.0	0.0	0.0	0.4
AKSSuT[c]	6.2	4.0	9.5	0.4	0.1	2.0
ACKSSuT[d]	5.0	2.9	8.5	0.0	0.0	0.4

[a] A3C-ampicillin, cefoxitin, ceftiofur, cephalothin

[b] ACSSuT-ampicillin, chloramphenicol, streptomycin, sulphamethoxazole and tetracycline

[c] AKSSuT-ampicillin, kanamycin, streptomycin, sulphamethoxazole and tetracycline

[d] ACKSSuT-ampicillin, chloramphenicol, kanamycin, streptomycin, sulphamethoxazole and tetracycline

Table 4.2. Prevalence (%) of AMR in calves sampled in the spring (n=480) and in the fall (n=395) of 2002 accounting for clustering of AMR within herd

Antimicrobial	Individual animal prevalence for calves in the spring			Individual animal prevalence for calves in the fall		
	Prevalence (%)	Lower CI	Upper CI	Prevalence (%)	Lower CI	Upper CI
Amikacin	0.0	0.0	1.0	0.0	0.0	1.2
Amoxicillin-Clavulanic Acid	7.1	4.4	11.2	0.0	0.0	0.0
Ampicillin	31.1	25.2	37.7	3.0	1.4	6.4
Cefoxtin	6.4	4.1	10.0	0.0	0.0	1.2
Ceftiofur	2.9	1.2	6.6	0.0	0.0	1.2
Ceftriaxone	0.0	0.0	1.0	0.0	0.0	1.2
Cephalothin	11.5	7.8	16.6	1.5	0.7	3.2
Chloramphenicol	22.3	16.6	29.2	0.8	0.3	2.3
Ciprofloxacin	0.0	0.0	1.0	0.0	0.0	1.2
Gentamicin	1.0	0.4	2.4	0.0	0.0	1.2
Kanamycin	28.9	22.9	35.8	2.2	0.9	5.4
Nalidixic Acid	0.2	0.03	1.5	0.0	0.0	1.2
Streptomycin	49.1	42.3	56.0	5.3	3.0	9.1
Sulphamethoxazole	56.3	49.6	62.7	7.3	4.8	11.0
Tetracylcine	60.0	53.3	66.4	9.9	6.2	15.5
Trimethoprim-Sulphamethoxazole	24.3	18.7	31.0	0.5	0.1	1.9
AMR (≥1 antimicrobial)	62.2	55.4	68.5	12.7	8.5	18.4
Multi AMR (≥2 antimicrobials)	59.3	52.6	65.6	9.9	6.4	15.2
A3C[a]	2.7	1.1	6.6	0.0	0.0	1.2
ACSSuT[b]	4.9	2.6	9.1	0.0	0.0	1.2
AKSSuT[c]	8.7	5.2	14.1	0.8	0.2	3.1
ACKSSuT[d]	10.0	6.7	14.6	0.0	0.0	1.2

[a] A3C-ampicillin, cefoxitin, ceftiofur, cephalothin

[b] ACSSuT-ampicillin, chloramphenicol, streptomycin, sulphamethoxazole and tetracycline

[c] AKSSuT-ampicillin, kanamycin, streptomycin, sulphamethoxazole and tetracycline

[d] ACKSSuT-ampicillin, chloramphenicol, kanamycin, streptomycin, sulphamethoxazole and tetracycline.

Table 4.3. Prevalence (%) of AMR at the herd level as determined by calves sampled in the spring (n=91) and in the fall (n=45) of 2002

Antimicrobial	Herd Prevalence for calves in the spring			Herd prevalence for calves in the fall		
	Prevalence (%)	Lower CI	Upper CI	Prevalence (%)	Lower CI	Upper CI
Amikacin	0.0	0.0	5.0	0.0	0.0	9.8
Amoxicillin-Clavulanic Acid	22.0	14.6	31.6	0.0	0.0	9.8
Ampicillin	62.6	52.3	71.9	17.8	9.2	31.7
Cefoxtin	1.1	0.2	7.4	0.0	0.0	9.8
Ceftiofur	8.8	4.5	16.6	0.0	0.0	9.8
Ceftriaxone	0.0	0.0	5.0	0.0	0.0	9.8
Cephalothin	31.9	23.1	42.1	13.3	6.1	26.7
Chloramphenicol	41.8	32.1	52.1	6.7	2.2	18.7
Ciprofloxacin	0.0	0.0	5.0	0.0	0.0	9.8
Gentamicin	5.5	2.3	12.5	0.0	0.0	9.8
Kanamycin	57.1	46.8	66.9	11.1	4.7	24.1
Nalidixic Acid	1.1	0.2	7.4	0.0	0.0	9.8
Streptomycin	80.2	70.8	87.2	26.7	15.8	41.3
Sulphamethoxazole	87.9	79.5	93.2	40.0	26.9	54.8
Tetracylcine	90.1	82.1	94.8	44.5	30.8	59.0
Trimethoprim-Sulphamethoxazole	48.4	38.3	58.6	4.4	1.1	16.1
AMR (≥1 antimicrobial)	91.2	83.4	95.5	55.6	41.0	69.2
Multi AMR (≥2 antimicrobials)	90.1	82.1	94.8	46.7	32.8	61.1
A3C[a]	7.7	3.7	15.3	0.0	0.0	9.8
ACSSuT[b]	13.2	7.6	21.8	0.0	0.0	9.8
AKSSuT[c]	26.4	18.4	36.4	4.4	1.1	16.1
ACKSSuT[d]	20.9	13.7	30.4	0.0	0.0	9.8

[a]A3C-ampicillin, cefoxitin, ceftiofur, cephalothin

[b]ACSSuT-ampicillin, chloramphenicol, streptomycin, sulphamethoxazole and tetracycline

[c]AKSSuT-ampicillin, kanamycin, streptomycin, sulphamethoxazole and tetracycline

[d]ACKSSuT-ampicillin, chloramphenicol, kanamycin, streptomycin, sulphamethoxazole and tetracycline

CHAPTER 5
PREVALENCE OF ANTIMICROBIAL RESISTANCE IN FECAL GENERIC *E. COLI* ISOLATED IN WESTERN CANADIAN BEEF HERDS. PART II: COWS AND COW-CALF PAIRS

5.1. Introduction

Antimicrobial resistance (AMR) in veterinary medicine is a complex issue. As in human medicine, there is concern about the loss of efficacious treatment options as a result of AMR. However, the bigger issue facing veterinarians and the livestock industry is the public health aspect of the issue because of evidence that agricultural use of antimicrobials contributes to increasing AMR in the human population. Several studies have investigated the potential link between antimicrobial use (AMU) and AMR in animals and the development of resistance in humans (Hummel et al., 1986, Endtz et al; 1991, Johnson et al., 1995, Bager et al., 1997, Aarestrup, 1999, Winokur et al., 2001, Swartz, 2002). Other studies of commensal and pathogenic resistant bacteria have been conducted in swine, poultry, feedlot, and dairy operations in order to more fully understand the type and level of resistance that is present in livestock (Dargatz et al., 2003, Fiztgerald et al., 2003, Lanz et al., 2003, Bywater et al., 2004, Rajic et al., 2004, Khachatryan et al., 2004, Hershberger et al., 2005).

Cow-calf herds in western Canada are subjected to less intensive management practices and different antimicrobial exposures than livestock species that are raised more intensively such as poultry, swine, or cattle in dairies or feedlots (Gow and

Waldner, 2007). The potential difference in selective pressure within cow-calf herds could, therefore, result in a different profile of AMR organisms when compared to other types of livestock. To the best of our knowledge there is currently no information on AMR in the cow-calf industry in western Canada. The objective of this study was to describe AMR patterns in cows and cow-calf pairs from western Canadian beef herds using *E. coli* as an indicator organism.

5.2. Materials and methods

As a part of a strategic research initiative to study AMR and AMU in cow-calf herds (Gow and Waldner, 2007, Gow et al., 2007a, Gow et al., 2007b), this project was undertaken to compliment an investigation of AMR in beef calves (Gow et al., 2007b). This paper describes AMR in generic fecal *E. coli* isolated from cows and cow-calf pairs. In the spring of 2002, feces were collected from a convenience sample of 533 individually identified cows that were accessible in the calving and nursery areas on 69 privately owned cow-calf farms in Alberta and Saskatchewan. In 2003, fecal samples were also collected from 105 cow-calf pairs on 10 farms. This analysis focuses on the above described population, but comparisons are made to other available data (Gow et al., 2007b) where calves had been sampled in the same herds.

The materials and methods utilized in this study have been described in detail elsewhere (Gow et al., 2007b). Briefly, fecal samples were obtained either directly from the rectum or from the ground immediately after defecation. A separate disposable glove and container were used for each animal. Fecal samples were cultured for generic

E. coli (Prairie Diagnostic Services, Saskatoon, Saskatchewan). A minimum of three isolates per sample identified as *E. coli* were selected and stored at -80°C. Isolates were tested for susceptibility using microbroth dilution (Sensititre®, TREK Diagnostic Systems Inc., Cleveland, Ohio) and the standard 2002 National Antimicrobial Resistance Monitoring System (NARMS) public health panel (CIPARS, 2006) (Agri-Food Laboratories Branch, Alberta Agriculture and Food, Edmonton, Alberta, Canada). All testing was done in accordance with NCCLS guidelines (NCCLS, 2000).

5.2.1. Statistical analysis

The approach utilized for data manipulation, and population averaged prevalence estimates have been described in detail elsewhere (Gow et al., 2007b). Models were developed using generalized estimating equations (GEE) to account for clustering within herd (SAS v.8.2 for Windows (PROC GENMOD); SAS Institute, Cary, North Carolina, USA). Model specifications included a binomial distribution, logit link function, repeated statement with subject equal to herd, and an exchangeable correlation structure. For the examination of unconditional associations between AMR and animal level risk factors of interest, the predictors included: cow breed, cow age (2 year old heifers, 3 year old cows, 4 to 10 year old cows, and cows >10 years of age), and whether the cow was ever treated in 2002 prior to sample collection. The AMR outcomes (yes/no) examined included resistance to sulphamethoxazole, to tetracycline, to any antimicrobial, or to multiple antimicrobials. The association between dam resistance status (yes/no) and calf resistance status (yes/no) was also examined using the model specifications outlined above.

The frequency of AMR was compared between calves and cows in the same herd. Generalized estimating equations using the above model specifications were used to compare the total number of samples with any type of AMR (numerator) as a proportion of the total number of animals sampled (denominator), first between cows and calves in the spring of 2002 and again in 2003, and then between cows in the spring of 2002 and calves in the fall of 2002. Only herds where both cows and calves were sampled were included in these analyses. There were 37 herds where both calf (Gow et al., 2007b) and cow samples were collected in the spring of 2002, and 10 herds from the present study where both calves and cows were sampled in the spring of 2003. There were nine herds where samples were collected from cows in the spring of 2002 and also from the calves in the fall of 2002 (Gow et al., 2007b).

In addition to examining the role of the cow-calf pair relationship in determining calf resistance status, we also investigated whether the most common resistance types found in the cow herd were a potential determinant of the types and frequency of resistance found in the calves. For the herds with both cow and calf samples in spring, we examined whether the proportion of cows with resistance to either sulphamethoxazole or tetracycline in the herd was a predictor of the proportion of calves in the herd (count of AMR positive calves / number of calves collected) with resistance to each of these antimicrobials respectively (Gow et al., 2007b), using GEE and the above model specifications.

5.3. Results

5.3.1. Study conducted in the spring of 2002

Cow age ranged from 2 to 14 years (median, 5 years; interquartile range (IQR), 3 to 8). Median herd size was 154 (range, 71 to 437) breeding females. The median number of samples collected per herd was 8 (range, 2 to10; IQR, 6 to 10). Before sample collection, producers reported that 4.1% (22/533) of cows had been treated with antimicrobials. The number of days between last treatment and sample collection ranged from 6 to 147 (median, 37).

5.3.2. Observed AMR in cows sampled in 2002

Resistance to at least one antimicrobial was identified in 9.8% of the 1555 isolates examined in 2002. At least one resistant isolate was identified in 15.1% of cows and 61% of herds (Tables 5.1 to 5.3). The antimicrobials to which resistance was most commonly identified were tetracycline and sulphamethoxazole (Tables 5.1 to 5.3). For all other drugs tested, the frequency of resistance was less than 2% (Tables 5.1 and 5.2). No resistance was identified to ceftriaxone, ciprofloxacin, and nalidixic acid.

The maximum number of antimicrobials to which an isolate demonstrated resistance was 11; 0.5% (7/1555) of isolates were resistant to at least five antimicrobials. The most common multi-resistance pattern found in this group of isolates included: ampicillin,

chloramphenicol, streptomycin, sulphamethoxazole, and tetracycline. Three of the seven isolates with resistance to five or more antimicrobials exhibited this pattern.

No resistance was detected to the Category I antimicrobials except for one isolate that had resistance to ceftiofur (Figure 5.1). The median MIC ranges for Category I antimicrobials were several dilutions away from the break point. With the exception of streptomycin, the median MICs for all Category II and III antimicrobials were also several dilutions below the breakpoint.

The detection of any AMR or multiple AMR was not associated with cow breed (P=0.16; P=0.11), cow age (P=0.14, P=0.42), or previous cow treatment (P=0.56, P=0.32). Tetracycline and sulphamethoxazole resistance were also not associated with cow breed (P=0.09, P=0.45), cow age (P=0.20, P=0.22), or previous cow treatment (P= 0.44, P=0.28).

5.3.3. Study of cow-calf pairs conducted in 2003

The median number of samples collected per herd was 10 (range, 9 to 16; IQR, 10 to 10), and median herd size was 130 (range, 86 to 382) breeding females. Cow age ranged from 2 to 19 years of age (median, 5 years; IQR, 3 to 8). Ninety-two percent of the cows were classified as healthy at the time of sample collection. Fifty-eight percent (61/105) of the calves sampled were male, and 91.4% (96/105) of the calves were classified as healthy at sample collection. Median calf age was 47 days (range, 1 to 129; IQR; 28 to 60).

5.3.4. Observed AMR in the cows from the cow-calf pairs sampled in 2003

Of the 312 isolates recovered from the cow samples in 2003, 6.1 % were resistant to at least one antimicrobial (Table 5.1); 8.6% of cows had at least one resistant isolate as did 60% of the herds (Table 5.2 to 5.3). Most of the resistance detected was to tetracycline and sulphamethoxazole.

The maximum number of antimicrobials that an isolate was resistant to was 7; 1.9% (6/312) of isolates demonstrated resistance to at least 5 antimicrobials. The most common pattern found in this group of isolates included: ampicillin, kanamycin, streptomycin, sulphamethoxazole, and tetracycline.

No resistance was identified to the Category I antimicrobials, and the median MIC ranges for these antimicrobials were several dilutions below the breakpoint (Figure 5.2). All of the median MICs for the Category II and Category III antimicrobials were several dilutions below the breakpoint (Figure 5.2), except for streptomycin which had a median MIC in the dilution immediately below the breakpoint.

5.3.5. Observed AMR in the calves from the cow-calf pairs sampled in 2003

Of the 318 calf isolates, 25.8 % were resistant to at least one antimicrobial (Table 5.1). The proportion of calves and herds with at least one resistant isolate were 37.9% and 100% respectively (Table 5.2 to 5.3). The majority of the resistance detected was to

tetracycline and sulphamethoxazole and no resistance was identified to ceftriaxone, nalidixic acid, and ciprofloxacin.

The maximum number of antimicrobials that an isolate was resistant to was 12; 9.1% (29/318) of isolates and 12.3% (13/105) of calves demonstrated resistance to at least five antimicrobials. The most common patterns found in this group of isolates included resistance to: ampicillin, choramphenicol, kanamycin, streptomycin, sulphamethoxazole, and tetracycline.

Ceftiofur was the only Category I antimicrobial to which isolates demonstrated resistance (Figure 5.3). The median MIC ranges for these Category I, Category II (except for streptomycin), and Category III antimicrobials were several dilutions below the breakpoint (Figure 5.3).

5.3.6. Observed AMR in the cow-calf pairs

Resistant *E. coli* were identified in both the cow and the calf for only 4.8% (5/105) of the pairs examined and 3 of 10 farms. Three of five resistant pairs had isolates resistant to two or more antimicrobials. Tetracycline was the most common drug resistance detected and was identified in four of five pairs. Calf resistance was not predicted by dam resistance (P=0.36).

5.3.7. Comparison of AMR prevalence between cows and calves

The prevalence of AMR was lower in generic fecal *E. coli* isolates collected from beef cows in the spring than in young calves from the same herds. In the 10 herds described above with cow-calf pair data for the spring of 2003, the calves were 7.1 (95% CI, 2.8 to 18.3; $P<0.0001$) times more likely to shed resistant isolates than the cows. A similar trend was detected for 37 herds that had samples collected in the spring of 2002 from both calves (Gow et al., 2007b) and cows. Calves were 10.0 (95% CI, 5.8 to 17.0; $P<0.0001$) times more likely to be positive for AMR than cows from the same herds. The median number of samples collected from the 37 herds (212 calves and 299 cows) was 13 (IQR, 12 to 17; range, 7 to 20).

There was no difference in the prevalence of AMR when comparing cows sampled in the spring of 2002 to calves sampled in the fall of 2002 (Gow et al., 2007b) from the same herds sampled (OR, 1.1; 95%CI, 0.3 to 3.7; $P=0.91$). The median number of samples from these 9 herds (81 calves and 74 cows) was 19 (IQR, 15 to 20; range, 9 to 20).

5.3.8. Association between the frequency of resistance in cow and calf samples

For the 37 herds that had both cow and calf samples collected in the spring of 2002, the odds that calves would be resistant to sulphamethoxazole increased with the proportion of cows that were resistant to sulphamethoxazole (OR, 7.5; 95% CI, 1.3 to 41.7; $P=0.02$). A similar increase in the odds of tetracycline resistance was seen with an

increasing proportion of cows positive for tetracycline resistance (OR, 6.1; 95% CI, 1.5 to 25.3; *P*=0.01).

5.4. Discussion

This study increases the knowledge of AMR in beef cattle by providing complementary data to a study of calves from cow-calf herds (Gow et al., 2007b). The prevalence of AMR is relatively low in cow populations particularly to drugs classified as important to human medicine by Health Canada. Beef cows in this study were much less likely to shed resistant organisms than very young calves; however, cows and calves have similar AMR prevalence by weaning. Other key findings of this study were that the individual cow is not the primary determinant of the AMR status of her calf, but that the frequency of common types of resistance in the calves is associated with exposure from the cow herd.

E. coli isolates collected from both the cows and calves were most commonly resistant to tetracycline and sulphamethoxazole. This pattern was explored by considering whether the proportion of cows in the herd with either tetracycline or sulphamethoxazole resistance was predictive of these same resistances in calves in the spring of 2002. The association between resistance in the cow herd and the occurrence of the same types of resistance in the calves indicates that young calves might be acquiring resistance by contact with the cow herd or fecal contamination of the environment by the cow herd. The data from the cow-calf pair study suggests that the

status of the calf's dam is less important than the herd environment in determining the calf's status.

The most common resistances detected in this study are consistent with what others have reported for *E. coli* isolates from a variety of different animal species (Kijima-Tanaka et al., 2003, Khachatryan et al., 2004, Bywater et al., 2004, Gow et al., 2007b). The difference in AMR prevalence between species (Schroeder et al., 2002, Kijima-Tanaka et al., 2003, Sayah et al., 2005) may be the result of variation in AMU selection pressure between industries. The perception is that in-feed antimicrobials are infrequently used in cow-calf herds and that there is often minimal routine injectable AMU. Currently there are limited data to supports this assumption; further work is necessary to understand the impact of selective pressures experienced in cow-calf herds.

The frequency of resistance was low in generic fecal *E. coli* isolates harvested from beef cows, especially to antimicrobials classified as important to human health. No ciprofloxacin or ceftriaxone resistance was present, and only one ceftiofur resistant isolate was identified from the cow samples. For the majority of the antimicrobials tested, the median MICs were also well below the breakpoint for resistance, indicating that most of the *E. coli* population in these animals was highly sensitive to those particular drugs. These findings indicate that on-farm exposure to beef cows probably poses a relatively low risk as a source of AMR for human health. However, additional molecular studies would provide more insight into what AMR genes are being carried

in these populations. Follow up monitoring is needed to detect emerging resistance issues in this population that could be of greater concern to public health.

To appreciate the impact on animal health research is needed to assess animal health pathogens in cow-calf herds and their AMR patterns. In an Alberta feedlot project some initial work has been performed on AMR in bovine respiratory pathogens (Read et al., 2004), but nothing is currently available from cow-calf herds. Access to clinical laboratory databases could provide some insight into AMR in animal pathogens, but often this information is incomplete and difficult to obtain. To assess the impact of AMR on animal health pathogens a prospective study to collect and test samples of interest from diseased animals prior to and after treatment, along with detailed treatment and outcome data would be needed.

There were slight differences in the AMR prevalence estimates between the 2002 and 2003 cow samples, but the antimicrobials to which resistance was detected were very similar. The confidence intervals for the two prevalence estimates overlap suggesting the difference was not significant. In the cow-calf pair study, only 105 animals on 10 herds were enrolled, while there were over 500 animals on 69 herds for the cow study. There were also differences in the prevalence estimates between the calves from the cow-calf pair study in 2003 (25.8%) and a larger sample of beef calves from a related study in the spring of 2002 (48.8%) (Gow et al., 2007b). But again, the antimicrobials to which resistance was detected was very similar in the two populations. The 2002 studies involved larger populations of cows and calves and were probably

more representative of western Canadian cow-calf herds. Additionally, the area covered by the 2002 studies was more geographically diverse and included more farms with varying management practices. The calves sampled in 2003 (median, 47 days) were also older on average than the calves sampled in 2002 (median, 6 days).

While the prevalence of AMR in the cows was significantly lower than that observed in young calves in the spring of the year, the prevalence estimates from cows were similar to those of older calves sampled in the fall. While these studies were not specifically designed to study the effect of age on the prevalence of AMR organisms in beef cattle, it does appear that there may be an age-related difference between the pre-ruminant calves and older animals in each study. The observation of relatively high levels of AMR in young animals has also been described by other researchers (Brophy et al., 1977, Hinton et al., 1984, Hinton, 1985, Mathew et al., 1999, Khachatryn et al., 2004, Gow et al., 2007b). Further work is necessary to describe the determinants of AMR in young calves. With the exception of a lower risk of AMR in calves less than 3 days of age, there were no individual animal risk factors identified for AMR in either calves or cows in either this or the previous study (Gow et al., 2007b). The results of the present study suggest that the calf's dam is the not the primary determinant of whether or not it sheds resistant organisms, but the association of AMR in the cow herd and in the calves may indicate that calves are acquiring AMR from either the cow herd itself or from contamination of the environment by the cow herd.

Seasonal variation of AMR in cow-calf herds could be examined by following cattle over time. The intention of the current study was to assess cattle in cow-calf herds at a time when animals are potentially under the highest stress. The calving season is also often the period when antimicrobial treatment may be most common because animals are most susceptible to disease as a result of crowding, confinement, stress associated with calving, and potentially adverse weather conditions.

The main limitation of this and the related calf study (Gow et al., 2007b) was the use of convenience samples rather than having a formal random sampling strategy for selecting herds and animals within herds. Access to the herds and the necessary calving and treatment records were provided through a larger study looking at factors affecting productivity in beef herds (https:/www.wissa.info). Substantial additional funding would have been required to run this as an independent study. Secondly, recruiting cow-calf herds for research during calving season is very difficult. Calving is an extremely busy time in commercial cow-calf operations and herd owners are reluctant to allow visitors for any purpose because of biosecurity concerns. Given the limitations of the available budget for laboratory analysis, we collected samples from as many herds as agreed to participate during this stressful period.

Random sampling of animals within the herd was also not practical. Herd owners could not be asked to provide access to all cows or calves to permit formal random sampling because of liability concerns associated with disease transmission due to crowding and handling and the potential for trauma related injuries in pregnant cows or

very young calves. Samples were collected from accessible animals on the day of a routine herd visit while trying to minimize any incursion on the herd owner's time. Any other approach to sample collection would have been met with immediate rejection by the majority of herd owners. However, this said, the potential for selection bias was low. Herd management as well as the risk of treatment and death loss in these herds was representative of what would be expected in moderate to large, commercial beef herds in western Canada (https:/www.wissa.info). Neither the herds nor animals sampled were chosen with any knowledge of the owners' AMU practices or AMR status.

These are the first available on-farm data describing the prevalence of AMR in beef cows in western Canada. The prevalence of resistance to drugs classified of high importance to human medicine by Health Canada was very low. These results suggest that AMR is relatively uncommon in beef cows at calving, but that cows shed a lower proportion of resistant bacteria as compared to their calves in the spring of the year. While it is unclear why the prevalence of AMR changes as the animal ages and after the summer pasture season, documenting this finding in cow-calf herds is important as baseline information is required as a first step in the development of any long-term monitoring and control programs.

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Figure 5.1. Minimum inhibitory concentrations for fecal generic *E. coli* isolates recovered from cows in the spring of 2002 arranged by Health Canada's classification of drugs (n=1555)

*	Antimicrobial	n	MIC Percentiles		Distribution (%) of MICs																
			Median	75th	<=0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	>512
	Ceftiofur	**1555**	**0.25**	**0.25**				5.9	73.7	20.1	0.2		0.1		**0.1**						
I	Ceftriaxone	**1555**	**<=0.25**	**0.25**					99.9					0.1							
	Ciprofloxacin	**1554**	**<=0.015**	**<=0.015**	99.7	0.3															
	Amikacin	**1555**	**1**	**2**						2.0	49.8	45.9	1.9	**0.4**							
	Amoxicillin-Clavulanic Acid	**1555**	**4**	**4**							3.3	23.1	69.2	4.0	0.2	0.1	**0.1**				
	Gentamicin	**1554**	**0.5**	**1**					19.3	44.5	35.5	0.6			0.1						
II	Kanamycin	**1555**	**<=8**	**<=8**										99.3	0.1			**0.6**			
	Nalidixic Acid	**1555**	**2**	**4**						0.1	3.2	51.4	44.5	0.7							
	Streptomycin	**1555**	**<=32**	**<=32**												96.5	2.3	**1.2**			
	Trimethoprim-Sulphamethoxazole	**1555**	**<=0.12**	**<=0.12**				92.7	4.9	1.9				**0.5**							
	Ampicillin	**1555**	**4**	**4**							5.0	38.5	51.7	4.1	0.1	0.1	**0.6**				
	Cefoxitin	**1555**	**4**	**4**							0.7	28.5	59.5	10.5	0.6	**0.2**					
III	Cephalothin	**1555**	**8**	**8**								0.7	18.3	60.7	20.1	0.1	**0.2**				
	Chloramphenicol	**1555**	**4**	**8**								5.5	59.5	33.6	0.7		**0.7**				
	Sulphamethoxazole	**1555**	**<=16**	**<=16**											92.8		0.1	0.1		0.1	**6.9**
	Tetracycline	**1555**	**<=4**	**<=4**									90.7	0.8	0.6	0.3	**7.6**				
IV																					

Note: Roman numerals I-III indicate the ranking of human importance, established by the Veterinary Drug Directorate, Health Canada. The unshaded fields indicate the range tested for each antimicrobial in the plate configuration. Vertical double bars indicate the breakpoints and highlighted cells locate the median. Numbers in bold font are the number of isolates with growth in all wells within the tested range, indicating the actual MIC is greater than that range of dilutions.

Figure 5.2. Minimum inhibitory concentrations for generic fecal *E. coli* isolates recovered from the cows of the cow-calf pair samples in the spring of 2003, arranged by Health Canada's classification of drugs (n=312)

*	Antimicrobial	n	MIC Percentiles		Distribution of Isolates (%) Across Minimum Inhibitory Concentrations (MIC) Ranges															
			Median	75th	<=0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	>512
I	Ceftiofur	312	0.25	0.25				2.6	79.2	18.3											
	Ceftriaxone	312	<=0.25	0.25					100.0												
	Ciprofloxacin	312	<=0.015	<=0.015	99.4	0.6															
II	Amikacin	312	1	2						1.3	50.3	46.8	1.6								
	Amoxicillin-Clavulanic Acid	312	4	4							3.5	25.6	65.4	3.8	1.6						
	Gentamicin	312	1	1					16.7	27.2	55.4	0.6									
	Kanamycin	312	<=8	<=8										97.1				2.9			
	Nalidixic Acid	312	2	4							2.2	64.7	32.1	1.0							
	Streptomycin	312	<=32	<=32												96.5	1.6	1.9			
	Trimethoprim-Sulphamethoxazole	312	<=0.12	<=0.12				92.3	5.4	0.3	0.3			1.6							
III	Ampicillin	312	4	4							4.5	35.6	50.6	7.4			1.9				
	Cefoxitin	312	4	4							0.3	35.9	48.7	15.1							
	Cephalothin	312	8	8								1.6	13.5	67.0	17.3	0.6					
	Chloramphenicol	312	4	8								3.8	61.2	32.7	2.2						
	Sulphamethoxazole	312	<=16	<=16											95.5						4.5
	Tetracycline	312	<=4	<=4									90.4	4.5			5.1				
IV																					

Note: Roman numerals I-III indicate the ranking of human importance, established by the Veterinary Drug Directorate, Health Canada. The unshaded fields indicate the range tested for each antimicrobial in the plate configuration. Vertical double bars indicate the breakpoints and highlighted cells locate the median. Numbers in bold font are the number of isolates with growth in all wells within the tested range, indicating the actual MIC is greater than that range of dilutions.

Figure 5.3. Minimum inhibitory concentrations for generic fecal *E. coli* isolates collected from the calves of the cow-calf pair samples in the spring of 2003, Health Canada's classification of drugs (n=318)

*	Antimicrobial	n	MIC Percentiles		Distribution of Isolates (%) Across Minimum Inhibitory Concentrations (MIC) Ranges															
			Median	75th	<=0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	>512
I	Ceftiofur	318	0.25	0.25				2.2	73.3	19.5	0.9			0.6	3.5						
	Ceftriaxone	318	<=0.25	0.25					95.0	0.6	0.3			1.3	1.9	0.9					
	Ciprofloxacin	318	<=0.015	<=0.015	100.0																
II	Amikacin	318	1	2						4.7	48.1	44.0	2.2	0.9							
	Amoxicillin-Clavulanic Acid	318	4	4							0.3	11.9	72.6	9.1	0.9	1.6	3.5				
	Gentamicin	318	1	1					23.0	6.0	70.4		0.3		0.3						
	Kanamycin	318	<=8	<=8										92.5			0.3	7.2			
	Nalidixic Acid	318	2	4							0.3	50.0	48.1	1.6							
	Streptomycin	318	<=32	<=32												85.5	6.3	8.2			
	Trimethoprim-Sulphamethoxazole	318	<=0.12	<=0.12				75.2	11.0	4.4	0.6			8.8							
III	Ampicillin	318	4	4							0.9	37.1	49.7	2.5			9.7				
	Cefoxitin	318	4	4							0.3	24.2	58.2	11.9	0.9	4.4					
	Cephalothin	318	8	16								0.6	6.0	56.3	30.2	1.9	5.0				
	Chloramphenicol	318	4	8								3.8	57.2	29.9			9.1				
	Sulphamethoxazole	318	<=16	>512											73.6						26.4
	Tetracycline	318	<=4	<=4									76.1	1.3		0.3	22.3				
IV																					

Note: Roman numerals I-III indicate the ranking of human importance, established by the Veterinary Drug Directorate, Health Canada. The unshaded fields indicate the range tested for each antimicrobial in the plate configuration. Vertical double bars indicate the breakpoints and highlighted cells locate the median. Numbers in bold font are the number of isolates with growth in all wells within the tested range, indicating the actual MIC is greater than that range of dilutions.

Table 5.1. Prevalence (%) of AMR in *E coli* isolates recovered from cows (n=1555) in the spring of 2002 and for cows (n=312) and calves (n=318) in the spring of 2003 adjusted for clustering at the herd level.

Antimicrobial	Cows 2002			Pair Cows 2003			Pair Calves 2003		
		95% Confidence Interval			95% Confidence Interval			95% Confidence Interval	
	Prevalence (%)	Lower	Upper	Prevalence (%)	Lower	Upper	Prevalence (%)	Lower	Upper
Amikacin	0.0	0.0	0.3	0.0	0.0	1.5	0.0	0.0	1.5
Amoxicillin-Clavulanic Acid	0.3	0.1	0.8	0.0	0.0	1.5	3.9	1.1	13.0
Ampicillin	0.7	0.2	2.3	2.0	0.3	12.0	8.7	4.1	17.7
Cefoxtin	0.2	0.0	0.8	0.0	0.0	1.5	3.2	0.7	13.1
Ceftiofur	0.1	0.0	0.4	0.0	0.0	1.5	2.9	0.6	13.8
Ceftriaxone	0.0	0.0	0.3	0.0	0.0	1.5	0.0	0.0	1.5
Cephalothin	0.3	0.1	0.8	0.7	0.1	4.1	6.0	2.7	12.7
Chloramphenicol	0.8	0.2	3.0	0.0	0.0	1.5	7.9	3.4	17.1
Ciprofloxacin	0.0	0.0	0.3	0.0	0.0	1.5	0.0	0.0	1.5
Gentamicin	0.2	0.1	0.6	0.0	0.0	1.5	0.3	0.0	2.0
Kanamycin	0.7	0.2	2.9	2.7	0.6	10.4	5.9	1.8	17.3
Nalidixic Acid	0.0	0.0	0.3	0.0	0.0	1.5	0.0	0.0	1.5
Streptomycin	3.4	1.2	9.3	3.4	1.1	9.7	13.0	7.2	22.3
Sulphamethoxazole	7.1	3.4	14.2	4.4	1.8	10.2	24.3	15.2	36.5
Tetracylcine	8.7	4.4	16.5	5.1	2.2	11.2	20.6	12.5	32.1
Trimethoprim-Sulphamethoxazole	0.5	0.2	1.7	1.7	0.3	10.1	7.3	2.7	18.2
AMR (≥1 antimicrobial)	9.8	5.1	18.2	6.1	3.1	11.7	25.8	16.6	37.8
Multi AMR (≥2 antimicrobials)	7.1	3.2	15.0	3.4	1.1	9.7	23.2	14.3	35.3
A3C[a]	0.1	0.0	0.4	0.0	0.0	1.5	2.9	0.6	13.8
ACSSuT[b]	0.1	0.0	0.4	0.0	0.0	1.5	0.9	0.3	3.5
AKSSuT[c]	0.1	0.0	0.4	2.0	0.3	12.0	1.2	0.3	5.0
ACKSSuT[d]	0.2	0.0	1.3	0.0	0.0	1.5	2.9	0.6	13.8

[a] A3C-ampicillin, cefoxitin, ceftiofur, cephalothin

[b] ACSSuT-ampicillin, chloramphenicol, streptomycin, sulphamethoxazole and tetracycline

[c] AKSSuT-ampicillin, kanamycin, streptomycin, sulphamethoxazole and tetracycline

[d] ACKSSuT-ampicillin, chloramphenicol, kanamycin, streptomycin, sulphamethoxazole and tetracycline

Table 5.2. Prevalence (%) of AMR in cows (n=533) sampled in the spring of 2002 and for cows (n=105) and calves (n=105) of the cow-calf pairs sampled in the spring of 2003 adjusted for clustering at the herd level.

Antimicrobial	Cows 2002			Pair Cows 2003			Pair Calves 2003		
	Prevalence (%)	95% Confidence Interval Lower	Upper	Prevalence (%)	95% Confidence Interval Lower	Upper	Prevalence (%)	95% Confidence Interval Lower	Upper
Amikacin	0.0	0.0	0.9	0.0	0.0	4.4	0.0	0.0	4.4
Amoxicillin-Clavulanic Acid	0.8	0.3	1.9	0.0	0.0	0.0	4.9	1.6	14.2
Ampicillin	1.5	0.8	2.9	2.0	0.3	11.8	11.7	5.7	22.3
Cefoxtin	0.6	0.2	1.7	0.0	0.0	4.4	4.9	1.6	14.2
Ceftiofur	0.2	0.0	1.3	0.0	0.0	4.4	3.8	0.9	14.3
Ceftriaxone	0.0	0.0	0.9	0.0	0.0	4.4	0.0	0.0	4.4
Cephalothin	0.8	0.3	1.9	0.9	0.1	6.0	10.5	5.9	17.9
Chloramphenicol	1.7	0.8	3.6	0.0	0.0	4.4	10.2	4.3	22.1
Ciprofloxacin	0.0	0.0	0.9	0.0	0.0	4.4	0.0	0.0	4.4
Gentamicin	0.4	0.1	1.5	0.0	0.0	4.4	0.9	0.1	6.0
Kanamycin	1.3	0.7	2.6	2.8	0.7	10.1	7.7	2.5	21.4
Nalidixic Acid	0.0	0.0	0.9	0.0	0.0	4.4	0.0	0.0	4.4
Streptomycin	6.7	4.5	9.9	4.8	2.1	10.5	20.7	12.4	32.6
Sulphamethoxazole	11.1	8.0	15.1	5.7	2.9	10.8	33.8	21.3	49.0
Tetracylcine	13.4	10.2	17.5	7.7	3.5	16.2	31.6	20.6	45.2
Trimethoprim-Sulphamethoxazole	1.7	0.7	4.3	2.0	0.3	11.8	9.2	3.6	21.2
AMR (≥1 antimicrobial)	15.1	11.7	19.3	8.6	4.3	16.3	37.9	25.3	52.4
Multi AMR (≥2 antimicrobials)	11.6	8.6	15.6	4.8	2.1	10.5	32.7	21.5	46.2
A3C[a]	0.2	0.0	1.3	0.0	0.0	4.4	3.8	0.9	14.3
ACSSuT[b]	0.2	0.0	1.3	0.0	0.0	4.4	1.9	0.5	6.5
AKSSuT[c]	0.2	0.0	1.3	2.0	0.3	11.8	2.1	0.7	6.3
ACKSSuT[d]	0.4	0.1	1.4	0.0	0.0	4.4	3.8	0.9	14.3

[a] A3C-ampicillin, cefoxitin, ceftiofur, cephalothin

[b] ACSSuT-ampicillin, chloramphenicol, streptomycin, sulphamethoxazole and tetracycline

[c] AKSSuT-ampicillin, kanamycin, streptomycin, sulphamethoxazole and tetracycline

[d] ACKSSuT-ampicillin, chloramphenicol, kanamycin, streptomycin, sulphamethoxazole and tetracycline

Table 5.3. Herd prevalence (%) of AMR for cows (N=69 herds) sampled in the spring of 2002 and for cows (N=10 herds) and calves (N=10 herds) of the cow-calf pairs sampled in the spring of 2003 adjusted for clustering at the herd level.

Antimicrobial	Cows 2002			Pair Cows 2003			Pair Calves 2003		
		95% Confidence Interval			95% Confidence Interval			95% Confidence Interval	
	Prevalence (%)	Lower	Upper	Prevalence (%)	Lower	Upper	Prevalence (%)	Lower	Upper
Amikacin	0.0	0.0	6.5	0.0	0.0	34.5	0.0	0.0	34.5
Amoxicillin-Clavulanic Acid	5.8	2.2	14.5	0.0	0.0	34.5	30.0	10.0	62.4
Ampicillin	11.6	5.9	21.5	10.0	1.4	46.7	50.0	22.5	77.5
Cefoxtin	4.4	1.4	12.6	0.0	0.0	34.5	30.0	10.0	62.4
Ceftiofur	1.5	0.2	9.6	0.0	0.0	34.5	20.0	5.0	54.1
Ceftriaxone	0.0	0.0	6.5	0.0	0.0	34.5	0.0	0.0	34.5
Cephalothin	5.8	2.2	14.5	10.0	1.4	46.7	70.0	37.6	90.0
Chloramphenicol	10.2	4.9	19.8	0.0	0.0	34.5	50.0	22.5	77.5
Ciprofloxacin	0.0	0.0	6.5	0.0	0.0	34.5	0.0	0.0	34.5
Gentamicin	2.9	0.7	10.9	0.0	0.0	34.5	10.0	1.4	46.7
Kanamycin	10.2	4.9	19.8	20.0	5.0	54.1	40.0	15.8	70.3
Nalidixic Acid	0.0	0.0	6.5	0.0	0.0	34.5	0.0	0.0	34.5
Streptomycin	34.8	24.5	46.7	40.0	15.8	70.3	80.0	45.9	95.0
Sulphamethoxazole	49.3	37.7	60.9	50.0	22.5	77.5	100.0	50.0	50.0
Tetracylcine	53.6	41.9	65.0	50.0	22.5	77.5	100.0	50.0	50.0
Trimethoprim-Sulphamethoxazole	8.7	4.0	18.0	10.0	1.4	46.7	40.0	15.8	70.3
AMR (≥1 antimicrobial)	60.9	49.0	71.6	60.0	29.7	84.2	100.0	N/A	N/A
Multi AMR (≥2 antimicrobials)	53.0	41.9	65.0	40.0	15.8	70.3	100.0	N/A	N/A
A3C[a]	1.5	0.2	9.6	0.0	0.0	34.5	20.0	5.0	54.1
ACSSuT[b]	1.5	0.2	9.6	0.0	0.0	34.5	20.0	5.0	54.1
AKSSuT[c]	1.5	0.2	9.6	10.0	1.4	46.7	20.0	5.0	54.1
ACKSSuT[d]	2.9	0.7	10.9	0.0	0.0	34.5	20.0	5.0	54.1

[a] A3C-ampicillin, cefoxitin, ceftiofur, cephalothin

[b] ACSSuT-ampicillin, chloramphenicol, streptomycin, sulphamethoxazole and tetracycline

[c] AKSSuT-ampicillin, kanamycin, streptomycin, sulphamethoxazole and tetracycline

[d] ACKSSuT-ampicillin, chloramphenicol, kanamycin, streptomycin, sulphamethoxazole and tetracycline

CHAPTER 6
FACTORS ASSOCIATED WITH ANTIMICROBIAL RESISTANCE IN CALVES BORN IN 89 WESTERN CANADIAN BEEF HERDS

6.1. Introduction

Antimicrobial use (AMU) in agriculture is a concern because of the potential for the spread of antimicrobial resistance (AMR) between animals and humans (Prescott and Dowling, 2000; McEwen and Fedorka-Cray, 2002, Anderson et al., 2003). Because AMU can lead to selection of resistant organisms, investigating the relationship between AMR and AMU is critical to understanding the risk factors associated with the development of resistance. The most commonly used antimicrobials in food animals are usually from one of five major classes: beta-lactams, tetracyclines, aminoglycosides, macrolides, and sulphonamides (White and McDermott, 2001). While all of these antimicrobials may not be "critical" antimicrobials in human medicine, the capacity of bacteria to carry multiple linked resistance genes may result in the transfer of unrelated resistance genes.

Since bacteria can carry multiple resistance genes on plasmids, transposons, and integrons it is necessary to investigate not only specific AMU/AMR combinations, but also to consider the effect of unrelated antimicrobials on the persistence of resistance in a population. For example, Read et al. (2002) demonstrated that the use of tetracycline,

florfenicol, and tilimicosin in feedlot cattle was associated with of the presence of the beta-lactamase enzyme *bla*$_{cmy2}$ gene. This finding suggests that AMU can select not only for resistance to that specific antimicrobial, but that it may also result in co-selection of other resistance genes.

In examining the risk factors for resistance development in a population, it is necessary to consider AMU in all members of the population and not just the animals from which samples have been collected. Antimicrobial use in some individuals can increase the risk of colonization or infection with resistant organisms in others who have not been treated. Members of a population can experience indirect effects of AMU including an increased risk for acquiring a resistant organism because of AMU in others in the population (Lipsitch and Samore; 2002). In environments with long-term AMU there will be a change in the ecology and resistant organisms will gain dominance within the population (White and McDermott, 2001).

The study objective was to investigate herd-level treatment and vaccination practices potentially associated with AMR in fecal generic *E. coli* collected from calves in beef herds from western Canada. Risk factors of potential interest included AMU in the herd, proportion of calves ever being treated, herd vaccination status for infectious bovine rhinotracheitis (IBR) and bovine virial diarrhea virus (BVDV), and vaccination status for calf-associated diarrhea cause by *E. coli*, rotavirus or coronavirus.

6.2. Materials and methods

6.2.1. Background and herd selection

In the spring of 2002, fecal samples were collected from calves born on western Canadian cow-calf herds. Participating herds were a subset of herds recruited for a multifaceted survey of risk factors affecting cattle productivity and health (https://www.wissa.info). Private veterinary clinics across northern British Columbia, Alberta, and Saskatchewan were approached and asked to participate in the larger study. Within each practice, herds were identified and enrolled based on the selection criteria which considered herd size (>50 cows), animal identification, existing calving records, animal handling facilities sufficient for pregnancy testing and bull evaluation, and relationship with a local veterinary clinic. Participating herds were visited at least quarterly by one of six study veterinarians to collect samples and data, and to monitor the quality and consistency of on-farm records. In a subset of herds, fecal samples and farm records for the current risk factor study were collected between January and June, 2002.

Calving records for each cow included cow identification, calf identification, date of calving, single or twin birth, sex of the calf, the degree of assistance provided to the cow, any post calving problems, and calving outcome (born alive, stillbirth, died later). If the calf died, the date of death was also included. Other data recorded for each herd included the herd vaccination status for infectious bovine rhinotrachietis (IBR), bovine

viral diarrhea virus (BVDV), and neonatal diarrhea (coronavirus or rotavirus, and *E. coli*). Herd inventory was monitored and tracked closely by study participants.

6.2.2 Antimicrobial use data collection

Antimicrobial use data were collected using individual treatment records as well as a questionnaire examining herd AMU (Gow and Waldner, 2007). Because the individual animal records did not consistently include information on the type of antimicrobial used for treatment, a questionnaire was developed to identify the types of antimicrobial products most commonly used on each cow-calf farm for the period of January 1 to June 30, 2002. Herd owners were asked about the frequency of use for sulphonamides, tetracylcines / oxytetracyclines, trimethoprim / sulphadioxine, and penicillins. Antimicrobials that did not fall into these broad categories were classified as “other”. Lists of common trade names were provided under each group to simplify the selection of the appropriate antimicrobial by the producer. Producers were asked to report separately the number of treatments for both cows and calves for each antimicrobial category listed above. The numbers of treatments for each category were coded as follows: l to 3 animals treated, 4 to 10 animals treated, and >10 animals treated. Only herd data were considered in investigating associations between AMU and AMR.

For each herd individual animal treatment data were summarized in order to assess the impact of the proportion of calves ever treated (yes/no) with antimicrobials and/or fluids on AMR. Treatment occurrence was reported for calves as cumulative incidence. The number of calves reported as ever having been treated as a percentage of the

number of calves in the herd at risk of treatment during the study period was used for this calculation.

6.2.3. Sample collection

Fecal samples were collected from 466 individually identified animals on 89 privately owned farms in Alberta and Saskatchewan. The calf samples were collected from accessible animals in the calving and nursery areas. Fecal samples were obtained either directly from the rectum or from the ground immediately after defecation. A separate disposable glove and container were used for each animal.

6.2.4. Laboratory methods

6.2.4.1 *Escherichia coli* culture

Fecal samples were sent on ice to a private diagnostic laboratory (Prairie Diagnostic Services, Saskatoon, Saskatchewan) for culture. The samples were cultured onto MacConkey agar plates at 37°C for 18 hours for isolation of *E. coli*. At least three individual lactose fermenting colonies identified as *E. coli* using standard biochemical tests, including indol, triple sugar iron (TSI) slant, citrate, and urea, were saved from each sample. If both dry and mucoid colonies were detected within a sample, then three isolates from each colony type were tested. Individual *E. coli* isolates were stored in 50% glycerol and Luria-Bertani (LB) broth at -80ºC until susceptibility testing was performed.

6.2.4.2. Susceptibility testing methodology

Susceptibility testing was performed by Alberta Agriculture, Food and Rural Development. All *E. coli* isolates were tested for susceptibility using a microbroth dilution technique (Sensititre®, TREK Diagnostic Systems Inc., Cleveland, Ohio) and the standard 2002 National Antimicrobial Resistance Monitoring System (NARMS) CMV7CNCD gram negative public health panel.

Minimum inhibitory concentrations (MICs) for a total of 16 antimicrobial agents were assessed (Figure 1). Breakpoints for susceptibility were used, as defined by the NCCLS (NCCLS, 2000) (Figure 2). All isolates that fell into the intermediate susceptibility range were classified as susceptible. Amikacin results > 4µg/mL were labeled not interpretable because the breakpoint is 4 dilutions beyond the range of the panel. The breakpoint used for streptomycin was 64µg/ml (CIPARS, 2006).

6.2.5. Statistical analysis

All data were entered into a computerized database (Microsoft® Office Access 2000, Microsoft Corporation). Descriptive analyses were completed and variables were recoded as necessary for statistical modeling using commercially available software programs (SPSS 11.0 for Windows, SPSS Inc., Chicago, Illinois).

Herd risk factors for AMR were investigated using generalized estimating equations (GEE) to account for clustering within herd (SAS v.8.2 for Windows (PROC GENMOD); SAS Institute, Cary, North Carolina, USA). Model specifications included a binomial distribution, logit link function, and an exchangeable correlation structure. Statistically significant associations were reported as an odds ratio with the lower and upper 95% confidence interval.

Separate models were run for each outcome of interest including the proportion of calves with resistance to: tetracycline, ampicillin, streptomycin, sulphamethoxazole, trimethoprim/sulphamethoxazole, kanamycin, chloramphenicol, ≥1 antimicrobial, and resistance to ≥2 antimicrobials. The numerator for each outcome was the number of positive calves for the herd and the denominator was the number of calves sampled from that herd.

In the first step of the analyses, the unconditional association between each of the outcomes of interest and the individual antimicrobials listed in tables 2 and 3 were investigated. Antimicrobial use was first modeled as a yes/no variable indicating whether the antimicrobial was used in the herd or not. If a statistically significant association was detected at a $P \leq 0.05$ level for the outcome and the use of that antimicrobial, then AMU was considered separately for cows and calves.

If a statistically significant association at ever used in the cows or calves on the farm was detected, then the analysis was repeated to consider the number of times the

antimicrobial was used in either cows or calves. The relationship between resistance and the increasing use of a particular antimicrobial was explored to determine if the odds of resistance increased with the number of times an antimicrobial was reported as used on the farm i.e. used 1-3 times, 4-10 times or >10 times. If a reasonable dose-response relationship was evident, then this categorization of the AMU was considered for use in building a multivariable herd-adjusted model. If the multivariable herd-adjusted model would not converge with the AMU categorized by use 1-3 times, 4-10 time or >10 times, then a second model was developed to evaluate a yes/no variable indicating any use of that antimicrobial for either cows and/or calves in the herd as appropriate. Again if this new model did not converge, then a summary yes/no variable indicating any use of the antimicrobial in the herd was evaluated in the model.

The final multivariable model was developed using backwards stepwise elimination. Any potential risk factors where $P \leq 0.05$ or that were acting as important confounders (removal of the potential risk factor from the model changes the effect estimate for the exposure by ≥20%) were retained in the final model. After establishing the main effects model, biologically reasonable first order interaction terms were tested if two or more variables ($P \leq 0.05$) were retained in the final model.

6.2.6. Post hoc power calculations

Post hoc power calculations were performed to evaluate whether limited study power was a factor in not detecting associations between specific AMU and AMR to that antimicrobial or class of antimicrobials. The most commonly reported antimicrobial

used and resistance detected was for tetracycline, therefore sample size estimates were based on this antimicrobial. The calculations were based on being able to detect a minimum expected increase of 1.5 times in the proportion of tetracycline resistance in calves from herds that used tetracycline compared with calves from herds that did not use tetracycline. Computations were performed using sample size calculations for comparing two proportions (Dohoo et al., 2003) to develop crude estimates of the required sample size per group. Data from a null model were used to estimate variance for AMR in calves across herds using penalized quasi-likelihood estimates (2^{nd} order PQL) (MLwiN version 2.0, Centre for Multilevel Modeling, Institute of Education, London, UK), a binomial distribution, and logit link function. These results were utilized in adjusting the sample size estimates for clustering at the herd level (Dohoo et al., 2003).

6.3. Results

6.3.1. Study population

Between 1 and 12 calf fecal samples (median, 5; interquartile range (IQR), 4 to 6) were collected from each of 89 herds. Enrolled herds ranged in size from 74 to 393 breeding females (median, 137). Of the 466 calves sampled, 56% (259/466) were male and ranged in age from new born to 151 days (median, 6). Dam age ranged from 2 to 17 years with a median of 6 years of age. The percentage of calves treated on each farm varied from 0 to 100% (median, 8.1%, IQR, 4.1 to 20.9%); 94% (440/466) of calves were classified as healthy at the time of sample collection.

Modified-live BVDV and IBR vaccines were used in 45% (40/89) of herds, 37% (33/89) of herd owners used an inactivated vaccination, 3% (3/89) vaccinated but the type was not reported, and 15% (33/89) did not vaccinate. Vaccination of the cow/heifers for prevention of neonatal calf diarrhea was reported in 32% (28/89) of herds.

6.3.2. Summary of AMR and AMU in study herds

The MICs for 16 antimicrobials were summarized for each of the 1677 isolates recovered in this study (Figure 6.1). For the 7 antimicrobials that resistance was most commonly detected, the crude calf prevalence ranged from 22.5 to 60.5% (Table 6.1). For the same 7 antimicrobials the median proportion of calves with AMR per herd was 21.0% to 59% (Table 6.1).

Ninety-one percent of herds had at least one calf positive for resistance to ≥1 antimicrobial. Similarly, resistance to ≥2 antimicrobials was detected in 89% of the herds. The most common resistances identified on farms were to tetracycline (90% of herds), sulphamethoxazole (88%), and streptomycin (80%).

More than 70% of the farms used tetracyclines/oxytetracylcines; the majority of this use was in the cow herd (Table 6.2). Slightly fewer than 70% of the herds used sulphonamides, but the majority of this use was in the calf herd (Table 6.2). Penicillin, tilmicosin and florfenicol were used on about half of the farms (Table 6.2 and 6.3).

Gentamicin, ceftiofur, and sulbactam:ampicillin were used by less than 12% of the herds. One percent of the herds reported off label use of either cephalexin or enrofloxacin (Table 6.3). Ionophores were incorporated into the rations of cows and heifers in 25.8% (23/89) herds.

6.3.3. Observed risk factors associated with AMR

Vaccination status for BVDV was not associated with resistance to streptomycin (*P*=0.58), sulphamethoxazole (*P*=0.37), trimethoprim/sulphamethoxazole (*P*=0.20), kanamycin (*P*=0.96), chloramphenicol (*P*=0.82), ampicillin (*P*=0.29), ≥1 antimicrobial (*P*=0.53), or ≥2 antimicrobials (*P*=0.55). Vaccination status of either heifers or cows, respectively, for calf associated diarrhea was also not associated with resistance to streptomycin (*P*=0.61, *P*=0.61), sulphamethoxazole (*P*=0.32, *P*=0.32), trimethoprim/sulphamethoxazole (*P*=0.16, *P*=0.14), kanamycin (*P*=0.57, *P*=0.61), chloramphenicol (*P*=0.09, *P*=0.09), ampicillin (*P*=0.84, *P*= 0.79), ≥1 antimicrobial (*P*=0.17, *P*=0.16), or ≥2 antimicrobials (*P*=0.31, *P*=0.30) . Ionophore use in the herd was not associated with resistance to streptomycin (*P*=0.77), sulphamethoxazole (*P*=0.40), tetracycline (*P*=0.56),trimethoprim/sulphamethoxazole (*P*=0.84), kanamycin (*P*=0.66), chloramphenicol (*P*=0.48), ampicillin (*P*=0.65), ≥1 antimicrobial (*P*=0.42), or ≥2 antimicrobials (*P*=0.61).

There were, however, several statistically significant unconditional associations between AMU and resistance to tetracycline (Table 6.4), streptomycin (Table 6.5), sulphamethoxazole (Table 6.6), trimethoprim/sulphamethoxazole (Table 6.7),

kanamycin (Table 6.8), chloramphenicol (Table 6.9), ampicillin (Table 6.10), ≥1 antimicrobial (Table 6.11), or ≥2 antimicrobials (Table 6.12).

In the final multivariable model for tetracycline resistance, any sulbactam:ampicillin use in the herd increased the odds of resistance 2.8 (95% CI, 1.0 to 7.4; *P*=0.04) times and the use of gentamicin in calves increased the odds of resistance 3.5 times (95% CI, 2.4 to 4.8; *P*<0.0001).

Sulbactam:ampicillin (OR, 3.2; 95% CI, 1.3 to 7.8; *P*=0.01) and gentamicin (OR, 5.5; 95% CI, 4.0 to 7.7; *P*<0.0001) use in calves were associated with an increased odds of streptomycin resistance in the final multivariable model.

The use of sulbactam:ampicillin in cows (OR, 6.1; 95% CI, 1.1 to 35; *P*=0.04) and gentamicin use in calves (OR, 3.4; 95% CI, 1.6 to 2.2; *P*<0.0001) was associated with the occurrence of sulphamethoxazole resistance in the final multivariable model .

The odds of trimethoprim/sulphamethoxazole resistance were 2.3 (95% CI, 1.1 to 5.0; *P*=0.03) times higher in herds that used any sulbactam:ampicillin than herds that did not. For every incremental increase in the proportion of calves treated in a herd, trimethoprim/sulphamethoxazole resistance also increased by 6.1 (95% CI, 1.5 to 25; *P*=0.01) times.

The final model of kanamycin resistance contained several risk factors. Kanamycin resistance was 3.9 (95% CI, 1.6 to 9.3; *P*=0.002) times more frequent in herds that used sulbactam:ampicillin in calves and 28.2 (95% CI, 4.8 to 166; *P*=0.0002) times more common in herds that used sulbactum:ampicillin in cows than herds that did not use sulbactum: ampicillin in calves or cows respectively. Kanamycin resistance was 6.2 (95% CI, 4.1 to 9.3; *P*<0.0001) times more likely in herds that used genatmicin in calves than in herds that did not use gentamicin in calves.

Only one risk factor was associated with chloramphenicol resistance. Resistance to chloramphenicol was 2.7 (95% CI, 1.2 to 6.0; *P*=0.02) times more frequent on farms that used florfenicol in calves.

Ampicillin resistance was 3.0 (95% CI, 1.5 to 6.1; *P*=0.002) times more likely in herds that used sulbactam:ampicillin in their calves and 5.3 (95% CI, 2.1 to13; *P*=0.0003) times more frequent in herds that used ceftiofur in calves than in herds that did not use either of these antimicrobials. The occurrence of ampicillin resistance was also 3.2 (95% CI, 2.3 to 4.5; *P*<0.0001) times more likely in herds that used enrofloxacin in calves than herds that did not use enrofloxacin in calves.

Resistance to ≥1 antimicrobial was associated with sulbactam:ampicillin use and gentamicin use in calves. Resistance to ≥1 antimicrobial was 3.1 (95% CI, 1.1 to 8.8; *P*=0.03) times more likely in herds that used any subactam:ampicillin and 3.2 (95% CI, 2.2 to 4.5; *P*<0.0001) times more likely in herds that used any gentamicin in calves.

Resistance to ≥2 antimicrobials in herds with any sulbactam:ampicillin use was 3.2 (95% CI, 1.2 to 8.6; P=0.02) times greater than herds that did not use sulbactam:ampicillin and 3.6 (95% CI, 2.5 to 5.0; P<0.0001) times greater in herds that used gentamicin in calves then herds that did not use gentamicin in calves.

6.3.4. Post hoc power calculations

The use of a specific antimicrobial was not commonly associated with resistance to that same antimicrobial or class of antimicrobials for some of the most prevalent resistances detected. For example, tetracycline resistance was not related to tetracycline/oxytetracycline use, sulphonamide use was not a risk factor for sulphamethoxazole or trimethoprim/ sulphamethoxazole resistance, and ampicillin resistance was not associated with penicillin use. The reason for this apparent discrepancy may be due to limited power in the analysis associated with restricted variation between herds in the frequency of both resistance and AMU for most of the above antimicrobials.

Tetracyline was used for the sample size calculations. In this population there were 57.5% (73/127) animals that were positive for tetracycline resistant isolates on farms with no tetracycline use. For farms with tetracycline use there were 61.7% (209/339) animals with tetracycline resistance. The difference in the percentage of animals with tetracycline resistance on farms with or without tetracycline use was only 1.1

(61.7/57.5) times. To adjust the sample size for clustering *rho* (0.87) was calculated for calves across herds.

Post hoc adjusted sample size calculations based on tetracycline and collecting 5 samples per herd, indicated that at least 190 animals would be needed per group to detect an odds ratio of 1.5. The current study had only 127 animals in herds that did not use any tetracycline, therefore the study lacked sufficient power to detect a minumum risk of 1.5 times between the study groups.

6.4. Discussion

This is one of the first studies to investigate factors associated with the frequency of AMR in fecal generic *E. coli* isolated from beef calves in cow-calf herds. The use of two antimicrobials, sulbactam:ampicillin and gentamicin, were identified as risk factors for the occurrence of resistance to several unrelated antimicrobials. These findings can potentially be explained by considering known mechanisms of AMR and the potential for co-selection of resistance genes.

Bacteria have a variety of methods to facilitate the promotion and transfer of resistance genes. Resistance genes can encode resistance not just to a particular antimicrobial, but to an entire class of antimicrobials (McDermott et al., 2003; Catry et al., 2003). They can also encode resistance to compounds that are structurally diverse through cross-resistance (Catry et al., 2003), therefore resulting in resistance to a variety of antimicrobials. Additionally, mobile genetic elements can also often carry several

resistance genes which can confer resistance to multiple antimicrobials through the acquisition of a single mobile element (McDermott et al., 2003). As a result, the treatment with any one antimicrobial, where resistance to that antimicrobial is encoded on a bacteria carrying multiple resistance genes, could promote the selection of resistance to the other antimicrobials through gene linkage (Enne et al.; 2001, Catry et al.; 2003).

Plasmids are one mechanism used by bacteria to carry and spread multiple resistance genes. The associations between the use of certain classes of antimicrobials and resistance to a different class could be explained by isolates carrying multi-resistant plasmids. These plasmids could then be selected for by any number of different antimicrobials and perpetuate resistance to a variety of unrelated antimicrobials.

From the data available it is impossible to tell what type of plasmid or other genetic mechanism is being selected in this population of beef calves, but one possibility that could explain the associations between sulbactam:ampicillin use and resistance to unrelated antimicrobials is the presence of a bla_{cmy2} plasmid. Winokur et al. (2001) demonstrated that *Salmonella* carrying bla_{cmy2} plasmids also carry resistance genes for tetracycline, aminoglycosides, and sulphonamides. Winokur et al. (2001) went on to illustrate that the bla_{cmy2} plasmid can transfer between *Salmonella* and *E. coli,* and that these *E. coli* also had high rates of co-resistance to the following antimicrobials: gentamicin, tobramycin, streptomycin, tetracycline, trimethoprim/sulphamethoxazole, and chloramphenicol.

Allen and Poppe (2002) also demonstrated that a non-conjugative *bla*$_{cmy2}$ plasmid with resistance for ampicillin, cefoxitin, ceftiofur, cephalothin, streptomycin, sulfisoxizole and tetracycline originally detected in *Salmonella* could be transferred to *E. coli*. In this same study chloramphenicol, florfenicol, kanamycin, and neomycin resistance were also transferred to *E. coli* from *S. Ohio* and *S. typhimurium* (Allen and Poppe; 2002).

Considering these earlier findings, in the current study the presence of a AmpC-like Beta-lactamase *bla*$_{cmy2}$ plasmid carrying resistance to multiple antimicrobials could explain why the use of sulbactam:ampicillin is associated with resistance to tetracycline, sulphamethoxazole, streptomycin, trimethoprim/ sulphamethoxazole, ampicillin, and resistance to ≥2 antimicrobials in this population of beef calves.

In addition to the use of sulbactam:ampicillin, resistance to kanamycin, streptomycin, tetracycline, sulphamethoxazole, and to ≥2 antimicrobials was also associated with gentamicin use in calves. As described above, the *bla*$_{cmy2}$ plasmid can carry kanamycin and neomycin resistance which may confer cross resistance to gentamicin.

The association between kanamycin and streptomycin resistance and gentamicin use are also likely the result of cross resistance between aminoglycosides. Selection of sulphamethoxazole and tetracycline resistance with gentamicin use could be the result

of a multiple resistant plasmid containing aminoglycoside resistance genes and resistance genes for each of these antimicrobials respectively.

While the presence of a *bla*$_{cmy2}$ plasmid appears to correspond with the combination of antimicrobial uses found to be significant risk factors for a variety of resistances, it is not the only possible explanation for these findings. Any multiple resistant plasmid or other multiple resistant genetic element could be carrying non *bla*$_{cmy2}$ beta-lactam resistance genes or aminoglycoside resistance genes along with other AMR genes. This means that the use of sulbactam:ampicillin or gentamicin could select for resistance to any number of different antimicrobials. Molecular analysis would be necessary to determine what mechanisms of resistance are involved for these particular isolates. Since the extended spectrum cephalosporins are important in the treatment of human disease if the presence of a *bla*$_{cmy2}$ plasmid was detected in this population it could have an impact on the spread of *bla*$_{cmy2}$ associated resistances in both people and animals. Continued monitoring would be needed to detect any potential rise in the AMR phenotypes associated with *bla*$_{cmy2}$.

In addition to the associations mentioned above, the study also detected an association between florfenicol use and chloramphenicol resistance. The relationship between florfenicol use and chloramphenicol resistance has also been described in feedlot cattle. Berge et al. (2005) demonstrated that immediately after treatment with florfenicol, all treated cattle shed isolates positive not only for chloramphenicol resistance, but for other antimicrobials as well.

In another feedlot study, Read et al. (2002) reported an association between ampicillin resistance and florfenicol use. This association was not detected in the calves of the current study. The current study did note an association between ampicillin resistance and both ceftiofur and sulabactum:ampicillin use, but these associations were not detected in the Read et al. (2002) study. The discrepancies between these two projects may be explained by a variety of factors including: methodological differences between these studies (fecal pat vs. swab fecal), animal age (young beef calves vs. feedlot beef calves post weaning), sampling differences (point in time vs. repeated sampling), methodological differences in sensitivity testing (microbroth dilution vs. agar dilution), and the level at which the analysis was performed (herd vs. individual). Finally and potentially most importantly, differences in AMU between cow-calf herds and feedlots may also have affected the outcomes.

While this investigation provides some of the first available data examining risk factors for beef calves, a recent study of commensal *E. coli* isolated from pre-weaned dairy calves on calf ranches and dairies described many factors associated with AMR (Berge et al, 2003; Berge et al, 2005). Farm type, animal source, calf age and individual treatments were important predictors of the odds of *E. coli* belonging to resistant clusters (Berge et al., 2006). Individual treatment with an injectable (yes/no) and/or oral (yes/no) AMU and calf age were investigated as risk factors, but were not associated with the occurrence of AMR in this group of beef calves (Gow et al., 2007).

One limitation of the current study was that detailed data on the type and dose of specific antimicrobials used were not gathered for individual animals, and therefore the relationship between specific antimicrobials and resistances could not be investigated at the individual animal level. A second limitation was that information on the frequency of herd AMU by producers was gathered retrospectively at the end of the calving season. Recall bias associated with retrospective data collection could result in potential over or under estimation of the number of times a particular antimicrobial was used in the herd or if it was used at all. Finally, because proportion of calves treated and AMU data were summarized for the entire period and not relative to the time of sample collection on that farm there is also misclassification bias. The herd might have been considered exposed to a certain antimicrobial or antimicrobials, but that exposure may have been subsequent to the sample collection. This same problem with time sequence would apply when considering the proportion of calves ever treated since many of the treatments could have taken place after sample collection, thereby having no relevance to the AMR patterns detected. Future studies could require individual treatment records that included the type of antimicrobial used, the date of use, and dose administered to more accurately determine individual animal exposure. However, historically detailed individual calf treatment records have been very difficult to obtain from most commercial cow-calf herds (Waldner, 2001)

Study power to investigate AMU/AMR associations was also limited. In these instances the lack of variability between farms made it impossible to investigate the association between resistance and AMU. Most of the herds enrolled in the study

routinely used tetracyclines, sulphonamides and penicillins. Many of these herds also had calves with AMR to the same antimicrobial. The lack of variability between farms in AMU and AMR detection meant that in order to study these associations that the numbers of animals in herds without tetracycline use would need to be increased to see a significant association if one was present.

This study does provide insight into whether treatment and vaccination practices influence AMR found in young calves in beef operations. This initial investigation suggests that because of the potential for linkage of unrelated resistance genes, we not only need to be aware of the risk of selecting for resistance to the antimicrobial being used, but we also need to consider that other resistance genes might be inadvertently selected and the potential impact that gene selection may have on both human and animals health.

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Figure 6.1. Minimum inhibitory concentration distribution for 1677 isolates from 466 calves tested for antimicrobial sensitivity using Sensititre 2002 NARMS CMV7CNCD plate configuration. Numbers are presented as a percentage of the total isolates (n=1677).

*	Antimicrobial	n	MIC Percentiles		Distribution (%) of MICs																
			Median	75th	<=0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	>512
I	Ceftiofur	1677	0.25	0.25				3.2	72.5	20.1	1.8	0.1	0.8	0.9	0.6						
	Ceftriaxone	1677	<=0.25	0.25					95.8	1.8	0.2	0.2	0.8	0.8	0.5	0.1					
	Ciprofloxacin	1677	<=0.015	<=0.015	99.4	0.4	0.1	0.2													
II	Amikacin	1677	2	2						0.5	29.6	65.1	4.4	0.4							
	Amoxicillin-Clavulanic Acid	1677	4	8							2.7	22.0	47.6	16.9	6.1	2.1	2.6				
	Gentamicin	1677	1	1					10.0	24.9	63.7	0.6		0.3	0.4	0.2					
	Kanamycin	1677	<=8	<=8										77.1	0.1			22.8			
	Nalidixic Acid	1677	4	4							0.8	30.6	65.9	2.5	0.1		0.2				
	Streptomycin	1677	<=32	64												62.6	20.9	16.5			
	Trimethoprim-Sulphamethoxazole	1677	<=0.12	0.5				52.6	14.7	12.0	0.9			19.8							
III	Ampicillin	1677	4	>=64							2.8	34.9	33.3	2.8	0.9	0.2	25.1				
	Cefoxitin	1677	4	4							0.2	20.6	56.0	17.4	1.6	4.2					
	Cephalothin	1677	8	16								1.0	13.4	57.5	21.0	1.6	5.5				
	Chloramphenicol	1677	8	8								2.1	39.6	39.1	1.4	0.2	17.5				
	Sulphamethoxazole	1677	<=16	>512											52.9	0.1		0.1		0.4	46.6
	Tetracycline	1677	8	>=64									49.1	1.1	0.2	1.6	48.0				
IV																					

Note: Roman numerals I-III indicate the ranking of human importance, established by the Veterinary Drug Directorate, Health Canada. The unshaded fields indicate the range tested for each antimicrobial in the plate configuration. Vertical double bars indicate the breakpoints and highlighted cells locate the median. Numbers in bold font are the number of isolates with growth in all wells within the tested range, indicating the actual MIC is greater than that range of dilutions.

Table 6.1. Antimicrobial resistance to any of the 7 antimicrobials to which resistance was most commonly detected, to any antimicrobial, or to ≥2 antimicrobials,were summarized as crude prevalence of AMR for calves (n=466) tested in all herds and as the median proportion of calves tested in each herd (IQR) (N=89)

	Crude calf prevalence	Herd prevalence
Antimicrobial		Median % (IQR)
Ampicillin	30.5% (142/466)	20.0% (0.0-50.0%)
Chloramphenicol	22.5% (105/466)	0.0% (0.0-38.0%)
Kanamycin	28.3% (132/466)	20.0% (0.0-50.0%)
Sulphamethoxazole	57.9% (270/466)	57.0% (33.0-80.0%)
Streptomycin	49.4% (230/466)	50.0% (20.0-80.0%)
Tetracycline	60.5% (282/466)	60.0% (33.0-80.0%)
Trimethoprim/sulphamethoxazole	24.5% (144/466)	0.0% (0.0-50.0%)
Any AMR	62.7% (292/466)	67.0% (40.0-90.0%)
AMR≥2 antimicrobial	60.1% (280/466)	60.0% (40.0-83.0%)

Table 6.2. The number (%) of herds using penicillins, sulphonamides, and tetracyclines/oxytetracycline (N=89)

Antimicrobial and cow or calf usage	# (%) of herds with no use	# (%) of herds with use (Y/N)	Number of times used on the farm		
			1-3 times	4-10 times	>10 times
Cow penicillin long acting	65 (73.0)	24 (27.0)	14 (15.7)	6 (6.7)	4 (4.5)
Cow penicillin short acting	79 (88.8)	10 (11.2)	4 (4.5)	3 (3.4)	3 (3.4)
Cow penicillin any	59 (66.3)	30 (33.7)	—	—	—
Calf penicillin long acting	82 (92.1)	7 (7.9)	4 (4.5)	—	3 (3.4)
Calf penicillin short acting	78 (87.6)	11 (12.4)	4 (4.5)	5 (5.6)	2 (2.2)
Calf penicillin any	74 (83.1)	15 (16.9)	—	—	—
Herd penicillin any	50 (56.2)	39 (43.8)	—	—	—
Cow oral sulphonamide	86 (96.6)	3 (3.4)	2 (2.2)	1 (1.1)	—
Cow injectable sulphonamide	81 (91.0)	8 (9.0)	7 (7.9)	—	1 (1.1)
Cow any sulphonamide	78 (87.6)	11 (12.4)	—	—	—
Calf oral sulphonamide	37 (41.6)	52 (58.4)	8 (9.0)	16 (18.0)	28 (31.5)
Calf injectable sulphonamide	59 (66.3)	11 (12.4)	6 (6.7)	13 (14.6)	30 (33.7)
Calf any sulphonamide	31 (34.8)	58 (65.2)	—	—	—
Herd any sulphonamide	29 (32.6)	60 (67.4)	—	—	—
Cow oxytetracycline LA	35 (39.3)	54 (60.7)	21 (23.6)	21 (23.6)	12 (13.5)
Cow oxytetracycline LP	86 (96.6)	3 (3.4)	2 (2.2)	1 (1.1)	—
Cow tetracycline bolus	87 (97.8)	2 (2.2)	1 (1.1)	1 (1.1)	—
Cow any oxytetracycline/tetracycline	33 (37.1)	56 (62.9)	—	—	—
Calf oxytetracycline LA	54 (60.7)	35 (39.3)	9 (10.1)	15 (16.9)	11 (12.4)
Calf oxytetracycline LP	88 (98.9)	1 (1.1)	—	1 (1.1)	—
Calf tetracycline bolus	88 (98.9)	—	—	1 (1.1)	1 (1.1)
Calf any oxytetracycline/tetracycline	53 (59.6)	36 (40.4)	—	—	—
Herd any oxytetracycline/tetracycline	26 (29.2)	63 (70.8)	—	—	—

Table 6.3. The number (%) of herds using tilmicosin, florfenicol, sulbactam:ampicillin, ceftiofur, enrofloxacin, gentamycin, amprolium and cephalexin (N=89)

Antimicrobial and cow or calf usage	No Use	Used (Y/N)	Number of times used on the farm		
			1-3 times	4-10 times	>10 times
Cow tilmicosin	79 (88.8)	10 (11.2)	9 (10.1)	1 (1.1)	—
Cow any tilmicosin	79 (88.8)	10 (11.2)	—	—	—
Calf tilmicosin	65 (73.0)	24 (27.0)	12 (13.5)	5 (5.6)	7 (7.9)
Calf any tilmicosin	65 (73.00	24 (27.0)	—	—	—
Herd any tilmicosin	57 (64.0)	32 (36.0)	—	—	—
Cow florfenicol	83 (93.3)	6 (6.7)	—	—	—
Calf florfenicol	47 (52.8)	42 (47.2)	—	—	—
Herd any florfenicol	45 (50.6)	44 (49.4)	—	—	—
Cow any sulbactam:ampicillin	87 (97.8)	2 (2.2)	—	—	—
Calf sulbactam:ampicillin	81 (91.0)	8 (9.0)	4 (4.5)	—	4 (4.5)
Calf any sulbactam:ampicillin	81 (91.0)	8 (9.0)	—	—	—
Herd any sulbactam:ampicillin	79 (88.8)	10 (11.2)	—	—	—
Cow any ceftiofur	88 (98.9)	1 (1.1)	—	—	—
Calf ceftiofur	82 (92.1)	7 (7.9)	1 (1.1)	2 (2.2)	4 (4.5)
Calf any ceftiofur	82 (92.1)	7 (7.9)	—	—	—
Herd any ceftiofur	82 (92.1)	7 (7.9)	—	—	—
Calf enrofloxacin calf	88 (98.9)	1 (1.1)	—	—	1 (1.1)
Herd enrofloxacin	88 (98.9)	1 (1.1)	—	—	—
Cow any mastitis	87 (97.8)	2 (2.2)	—	—	—
Cow any gentamicin	88 (98.9)	1 (1.1)	—	—	—
Calf any gentamicin	87 (97.8)	2 (2.2)	—	—	—
Herd any gentamicin	86 (96.6)	3 (3.4)	—	—	—
Calf amprolium	87 (97.8)	2 (2.2)	1 (1.1)	1 (1.1)	—
Calf any amprolium	87 (97.8)	2 (2.2)	—	—	—
Calf any cephalexin	88 (98.9)	1 (1.1)	—	—	—

Table 6.4. The statistically significant herd-adjusted unconditional association between AMU in the herd and the occurrence of resistance to tetracycline in *E.coli* isolates from beef calves (n=466, N=89)

Risk factor	Odds Ratio	95% Confidence Interval		*P*-value
		Lower	Upper	
Sulbactam:ampicillin				
Sulbactam:ampicillin used	2.7	1.0	7.2	0.04
No sulbactam:ampicillin used		Reference category		
Ceftiofur				
Any ceftiofur used in calves	3.0	2.1	4.2	<0.0001
No ceftiofur used in calves		Reference category		

Table 6.5. The statistically significant herd-adjusted unconditional association between AMU risk factors in the herd and the occurrence of calf resistance to streptomcyin (n=466, N=89)

Risk factor	Odds Ratio	95% Confidence Interval		*P*-value
		Lower	Upper	
Sulbactam:ampicillin				
Sulbactam:ampicillin used	3.0	1.3	7.0	0.01
No sulbactam:ampicillin used		Reference Category		
Sulbactam:ampicillin used in calves	3.0	1.3	7.4	0.01
No sulbactam:ampicillin in calves		Reference category		
Gentamicin				
Any gentomcyin used in calves	4.8	3.4	6.6	<.0001
No gentamicin used in calves		Reference category		
Cephalexin				
Any cephalexin used in calves	4.2	3.1	5.6	<.0001
No cephalexin used in calves		Reference category		
Other				
Other antimicrobials used in calves	4.1	2.4	6.8	<.0001
No other antimicrobials used in calves		Reference category		

Table 6.6. The statistically significant herd-adjusted unconditional association between AMU risk factors in the herd and the occurrence of calf resistance to sulphamethoxazole (n=466, N=89)

Risk factors	Odds Ratio	95% Confidence Interval		*P*-value
		Lower	Upper	
Sulbactam:ampicillin				
Sulbactam:ampicillin used	2.6	1.0	6.6	0.05
No Sulbactam:ampicillin used		Reference category		
Sulbactam:ampicillin used in cows	6.0	1.0	34.5	0.05
No sulbactam:ampicillin in cows		Reference category		
Gentamicin				
Any gentamicin used in calves	3.3	2.4	4.7	<.0001
No gentamicin used in calves		Reference category		
Other				
Any other antimicrobials used in calves	2.1	1.1	4.0	0.03
No other antimicrobials used on calves		Reference category		

Table 6.7. The statistically significant herd-adjusted unconditional association between AMU risk factors in the herd and the occurrence of calf resistance to trimethoprim/sulphamethoxazole (n=466, N=89)

Risk Factors	Odds Ratio	95% Confidence Interval		*P*-value
		Lower	Upper	
Sulbactam:ampicillin				
Any sulbactam:ampicillin used	2.6	1.1	6.3	0.04
No sulbactam:ampicillin used		Reference category		
Ceftiofur				
Any ceftiofur used on farm	3.5	1.1	11.6	0.04
No ceftiofur used on farm		Reference category		
Any ceftiofur used in calves	3.5	1.1	11.6	0.04
No ceftiofur used in calves		Reference category		
Proportion of calves treated				
Total proportion of calves treated	7.2	1.4	36.2	0.02

Table 6.8. The statistically significant herd-adjusted unconditional association between AMU risk factors in the herd and the occurrence of calf resistance to kanamycin (n=466, N=89)

Risk Factor	Odds Ratio	95% Confidence Interval		*P*-value
		Lower	Upper	
Sulbactam:ampicillin				
Any sulbactam:ampicillin used on the farm	4.4	1.9	10.0	<.001
No sulbactam:ampicillin used on the farm		Reference category		
Any sulbactam:ampicillin used in the cows	21.5	3.7	125.6	<.001
No sulbactam:ampicillin used in the cows		Reference category		
Any sulbactam:ampicillin used in the calves	3.4	1.4	8.0	0.01
No sulbactam:ampicillin used in the calves		Reference category		
Gentamicin				
Any gentamicin used on the farm	3.0	1.3	6.9	<.0001
No gentamicin used on the farm		Reference category		
Any gentamicin used in the calves	4.6	3.1	6.9	<.0001
No gentamicin used in the calves		Reference category		
Other				
Any other antimicrobials used in the calves	3.0	1.2	7.1	0.01
No other antimicrobials used in the calves		Reference category		
Proportion of calves treated				
Proportion of calves treated	7.1	1.6	32.5	0.01

Table 6.9. The statistically significant herd-adjusted unconditional association between AMU risk factors in the herd and the occurrence of calf resistance to chloramphenicol (n=466, N=89)

Risk factor	Odds Ratio	95% Confidence Interval		*P*-value
		Lower	Upper	
Florfenicol				
Any florphenical used on farm	2.7	1.2	6.2	0.02
No florphenical used on farm		Reference category		
Any florfenicol used in calves	2.7	1.2	6.0	0.02
No florphenical used in calves		Reference category		
Ceftiofur				
Any ceftiofur used on the farm	4.0	1.0	16.4	0.05
No ceftiofur used on the farm		Reference category		
Any ceftiofur used in calves	4.0	1.2	16.4	0.05
No ceftiofur used in calves		Reference category		

Table 6.10. The statistically significant herd-adjusted unconditional association between AMU risk factors in the herd and the occurrence of calf resistance to ampicillin (n=466, N=89)

Risk Factor	Odds Ratio	95% Confidence Interval		*P*-value
		Lower	Upper	
Penicillin				
Any penicillin used on farm	2.0	1.1	3.7	0.04
No penicillin used on farm		Reference category		
Florfenicol				
Any florfenicol used on farm	1.9	1.0	3.6	0.04
No florfenicol used on farm		Reference category		
Any florfenicol used in calves	1.9	1.0	3.5	0.05
No florfenicol used in calves		Reference category		
Sulbactam:ampicillin				
Any sulbactam:ampicillin used on farm	2.5	1.1	6.0	0.04
No sulbactam:ampicillin used on farm		Reference category		
Any sulbactam:ampicillin used in calves	3.2	1.3	7.7	0.009
No sulbactam:ampicillin used in calves		Reference category		
Ceftiofur				
Any ceftiofur used on farm	5.6	1.9	16.5	0.002
No ceftiofur used on farm		Reference category		
Any ceftiofur used in cows	7.0	5.0	9.7	<.0001
No ceftiofur used in cows		Reference category		
Any ceftiofur used in calves	5.6	1.9	16.5	0.002
No ceftiofur used in calves		Reference category		
Enrofloxacin				
Any enrofloxacin used in calves	2.3	1.7	3.2	<.0001
No enrofloxacin used in calves		Reference category		
Cephalexin				
Any cephalexin used in calves	1.5	1.1	2.1	0.01
No cephalexin used in calves		Reference category		
Gentamicin				
Any gentamicin used in calves	2.3	1.7	3.2	<.0001
No gentamicin used in calves		Reference category		

Table 6.11. The statistically significant herd-adjusted unconditional association between AMU risk factors in the herd and the occurrence of calf resistance to any antimicrobial (n=466, N=89)

Risk factor	Odds Ratio	95% Confidence Interval		*P*-value
		Lower	Upper	
Sulbactam:ampicillin				
Any sulbactam:ampicillin used on farm	3.0	1.1	8.5	0.04
No sulbactam:ampicillin used on farm		Reference category		
Gentamicin				
Any gentamicin used in calves	2.7	1.9	3.9	<0.0001
No gentamicin used in calves		Reference category		
Cephalexin				
Any cephalexin used in calves	2.4	1.8	3.3	<0.0001
No cephalexin used in calves		Reference category		

Table 6.12. The statistically significant herd-adjusted unconditional association between AMU risk factors in the herd and the occurrence of calf resistance to two or more antimicrobials (n=466, N=89)

Risk factor	Odds Ratio	95% Confidence Interval		*P*-value
		Lower	Upper	
Sulbactam:ampicillin				
Any sulbactam:ampicillin used in cows	3.1	1.1	8.3	0.03
No sulbactam:ampicillin used in cows		Reference category		
Gentamicin				
Any gentamicin used in calves	3.1	2.2	4.3	<.0001
No gentamicin used in calves		Reference category		
Other				
Any other antimicrobials use in calves	2.6	1.5	4.3	0.0004
No other antimicrobials antimicrobials in calves		Reference category		
Cephalexin				
Any cephalexin used in calves	2.7	2.0	3.6	<.0001
No cephalexin used in calves		Reference category		

CHAPTER 7
MOLECULAR CHARACTERIZATION OF AMR IN FECAL GENERIC *ESCHERICHIA COLI* ISOLATES IN WESTERN CANADIAN COW-CALF HERDS: PART I ASSOCIATIONS BETWEEN PHENOTYPE AND GENOTYPE

7.1. Introduction

There are many different genetic determinants of antimicrobial resistance (AMR) and each determinant may present a different distribution among bacterial populations (Lanz et al., 2003). Typically, AMR is reported based only on the expressed phenotype derived from susceptibility testing of the organism. However, resistance phenotypes alone do not always represent all of the underlying resistance genes. Alternatively, the presence or absence of a resistance gene does not imply that the particular strain is resistant or susceptible to an antimicrobial (Aarts et al., 2006). Evaluating both phenotype and genotype together provides a more complete understanding of the epidemiology of AMR.

Bacteria are proficient at sharing genetic information necessary to survive in the presence of antimicrobials (McDermott et al. 2002). The ability to readily exchange genes increases the potential of the spread of AMR determinants from commensal organisms present in animals and people to veterinary or human pathogens (Salyers and Cuevas, 1997). Rapid transfer of resistance can happen within and between genera of bacteria (McDermott et al., 2002). Even the passage of an ingested resistant organism

through the intestinal tract can result in the transfer of resistant genes to resident microflora, which may subsequently be transmitted to pathogenic bacteria (McDermott et al. 2002).

The speed of resistance development is affected by the bacteria involved, the selective pressure from AMU, and the availability and transferability of resistance genes (Schwartz et al., 2006). Loss of acquired resistance is influenced mainly by selective pressure, but also by the co-location of the resistance genes in multi-resistance gene clusters or integron structures (Schwartz et al., 2006). When resistance genes are organized in gene clusters or integrons, the loss of acquired resistance genes may not occur even in the absence of direct selective pressure (Schwartz et al., 2006).

Molecular methods have helped determine the genetic basis for AMR and provide the means for understanding how resistance genes are acquired and transmitted among bacteria. These methods could also lead to novel approaches to limit AMR dissemination. The localization of AMR genes on plasmids or chromosomes suggest that genes conferring multiresistance can exist as complex configurations of physically linked elements (Carattoli, 2001). Many aspects of the development of AMR remain uncertain. It is known, however, that AMR is the result of numerous and complex interactions among antimicrobials, micro-organisms, and the surrounding environment (White and McDermott, 2001). These factors may vary between livestock species; therefore the investigation of resistance patterns within different livestock production

systems is necessary to continue developing a clearer understanding of AMR epidemiology.

While there is some information about AMR in swine (Dunlop et al., 1998, Rajic et al., 2006), dairy (Mackie et al., 1988) and feedlot cattle (Read et al., 2005) there are very little data for cow-calf herds. As a part of a larger study designed to look at calf health and productivity, AMR was examined for various age groups within cow-calf herds in western Canada. A subset of the *E. coli* isolates collected for susceptibility testing were selected for more intensive investigation including resistance gene testing. The objective of this study was to measure the associations between AMR phenotypes and resistance genes in 207 generic *Escherichia coli* isolates obtained from a study of 77 cow-calf herds.

7.2. Materials and methods

7.2.1. General aspects of the study and sample collection

Fecal samples were collected from 1407 individually identified animals on 148 privately owned beef farms in Alberta and Saskatchewan (Figure 7.1) (Gow et al., 2007a, Gow et al., 2007b). The farms were part of a larger survey for risk factors affecting calf health. Samples were collected from three study groups: (1) 480 calves and (2) 533 cows in the spring of 2002 and (3) 394 calves sampled in the fall of 2002. Where possible samples collected in the fall came from the same farms as samples collected in the spring. The spring samples were collected from accessible cows or

calves in the calving and nursery area. The fall samples were collected from calves prior to weaning and during fall processing. Fecal samples were obtained either directly from the rectum or from the ground immediately after defecation. A separate disposable glove and container were used for each animal sampled. Spring samples were collected from March to July and fall samples were collected between August and December.

7.2.2. Laboratory methods

7.2.2.1 *Escherichia coli* culture

Fecal samples were sent on ice to a diagnostic laboratory (Prairie Diagnostic Services, Saskatoon, Saskatchewan) for culture. The samples were cultured onto MacConkey agar plates at 37°C for 18 hours for isolation of *E. coli*. At least three individual lactose fermenting colonies from each sample were identified as *E. coli* using standard biochemical tests including indole, Triple Sugar Iron (TSI) slant, citrate and urea. If both dry and mucoid colonies were detected within a sample, then three isolates from each colony type were tested. Individual *E. coli* isolates were stored in 50% glycerol and Luria-Bertani (LB) broth at -80ºC until sensitivity testing was completed.

7.2.2.2. Susceptibility testing methodology

E. coli isolates were tested for susceptibility (Alberta Agriculture and Rural Development) using a microbroth dilution technique (Sensititre®, TREK Diagnostic

Systems Inc., Cleveland, Ohio) and the standard 2002 National Antimicrobial Resistance Monitoring System (NARMS) panel (CIPARS, 2006).

Minimum inhibitory concentrations (MICs) were assessed for 16 antimicrobial agents (Figure 7.1). Breakpoints for susceptibility were used, as defined by the National Committee on Clinical Laboratory Standards (NCCLS) (NCCLS, 2000) (Figure 7.2). All isolates that fell into the intermediate susceptibility range were classified as susceptible. Amikacin results > 4μg/ml were labeled not interpretable because the breakpoint is 4 dilutions beyond the range of the panel. The breakpoint used for streptomycin was 64μg/ml (CIPARS, 2006).

7.2.2.3. Methodology for detecting resistance genes

7.2.2.3.1. Selection of samples for genotype testing

Genetic testing was completed on 12.2% (134/1099) of all resistant isolates collected and 2.2% (73/3319) of all susceptible isolates (Figure 7.1). The isolates were divided into susceptible or resistant. Since genetic determinants of AMR were of interest the majority of isolates (65%, 134/207) selected for this project were classified as resistant phenotypically. From either the susceptible or resistant list, isolates were randomly selected ensuring that not ≥1isolate from the same animal was included; therefore, this subset of isolates represents 207 animals from 77 farms.

DNA hybridization and PCR were used to test for 24 resistance genes from 6 antimicrobial families (Département de Pathologie et Microbiologie, Faculté de Médecine Vétérinaire, Université de Montréal, Saint-Hyacinthe, Québec). The antimicrobial family, the genetic marker along with the PCR primer sequence, and source of DNA are summarized in Table 7.1.

7.2.2.3.2. Bacterial strains and growth conditions

The 28 strains used as positive controls and templates for DNA amplification were obtained from different laboratories (Maynard et al., 2003, Maynard et al., 2004). These strains were stored at -80°C in tryptic soy broth medium containing 10% glycerol (vol/vol) and were propagated on Luria-Bertani broth or agar containing one of the following antimicrobial agents at the appropriate concentrations: ampicillin (50 μg/ml), gentamicin (30 μg/ml), kanamycin (50 μg/ml), tetracycline (10 μg/ml), chloramphenicol (10 μg/ml), trimethoprim (10 μg/ml), and sulfamethazine (200 μg/ml).

7.2.2.3.3. Detection of antimicrobial resistance genes

Oligonucleotide primers for PCR amplification of AMR gene sequences are described in Maynard et al. (2003, 2004). Template DNA was prepared from bacterial cultures by the boiling method of Daigle et al. (1994). PCR reactions (total volume, 50 μl) contained 1x PCR buffer (10 mM Tris-HCl pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$ (Amersham Pharmacia Biotech Inc., Piscataway, N.J.), 200 μM each of the four

deoxynucleoside triphosphates, 1 U of Taq DNA polymerase (Amersham Pharmacia Biotech Inc.), 25 pmol of each primer and 5 μL of template. DNA amplification was carried out in a GeneAmp PCR system 9700 (Perkin-Elmer, Foster City, Calif.) with the following conditions: 5 min at 94°C, followed by 30 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 1.5 min. An aliquot (3 μL) of each PCR reaction was resolved in a 1.2% agarose gel to confirm product size and purity. PCR products were labeled with [α-^{32}P] dCTP by using Ready-To-Go DNA Labeling Beads (Amersham Pharmacia Biotech Inc.). Colony hybridizations were performed as described previously (Harel et al., 1991).

7.2.3. Statistical analysis

Descriptive analyses were completed using commercially available software (SPSS 11.0 for Windows, SPSS Inc., Chicago, Illinois). Initially all isolates were coded as to the presence or absence of each phenotype and resistance gene considered in the analysis (Table 7.2). Multiple AMR was defined as phenotypic resistance to ≥2 antimicrobials. Isolates were further categorized for the presence or absence of at least one gene for each of the six families of antimicrobials considered. For example, if an isolate contained any individual *tet* resistance gene or any combination of *tet* resistance genes it would have been classified as being positive for the individual genes respectively, but it also would have been classified as being tetracycline gene positive.

Antimicrobial resistance patterns consistent with the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS, 2006) program were investigated

for associations with resistance genes. The specific patterns included; A3C (ampicillin, cefoxitin, ceftiofur and cephalothin), ACSSuT (ampicillin, chloramphenicol, streptomycin, sulphonamides, tetracycline), AKSSuT (ampicillin, kanmycin, streptomycin, sulphonamides, tetracycline), and ACKSSuT (ampicillin, chloramphenicol, kanamycin, streptomycin, sulphonamides, tetracycline). These patterns are often used in reference to *Salmonella* spp. (Ihnot et al., 1998; Bolton et al., 1999; Casin et al., 1999; Schmieger and Schicklmaier, 1999) and though the mechanism of resistance carriage for these patterns may not be the same in *E. coli*, they do permit the reporting of common resistance patterns detected in *E. coli* using a standard nomenclature.

All four of the above patterns as well as individual antimicrobial phenotypes that contribute to the A3C, ACSSuT, AKSSuT, or ACKSSuT patterns were explored for any association with the resistance genes investigated (Table 7.2). If the proportion of isolates carrying a particular resistance gene was less than 1.5%, the gene was excluded from the analysis. Unconditional associations were analyzed using generalized estimating equations (GEE) to account for clustering within herd (SAS v.8.2 for Windows (PROC GENMOD); SAS Institute, Cary, North Carolina, USA). Model specifications included a binomial distribution, logit link function, repeated statement with subject equal to herd, and an exchangeable correlation structure.

Statistically significant associations were reported as odds ratios (OR) with 95% confidence limits. Odds ratios >1 indicate an increasing occurrence of the genotype

being studied with the phenotype being measured (a positive association) while those <1 indicate a decreasing occurrence of the genotype being studied with increases in the measured phenotype (a negative association). Multiple comparisons were accounted for using a Bonferroni correction to provide a conservative estimate for the level of statistical significance (Dohoo et al., 2003) An association was significant if $P<0.004$ after correction for 14 comparisons ($P < 0.05/k$, k = number of comparisons) (Dohoo et al., 2003).

The relationship between the number of antimicrobials to which an isolate was resistant and the number of resistance genes detected was analyzed using GEE to account for clustering within herd. Model specifications included a Poisson distribution, log link function, repeated statement with subject equal to herd, and an exchangeable correlation structure. Variables in the unconditional analysis were considered statistically significant at $P<0.05$.

7.3. Results

7.3.1. Description of the samples examined in the phenotype and genotype comparison study

The subset of 207 fecal generic *E. coli* isolates represented 6.4% (107/1677) of all isolates recovered from the calves tested in the spring of 2002, 3.2% (50/1555) of isolates from the cows tested in the spring of 2002, and 4.2% (50/1186) of isolates from the calves tested in the fall of 2002.

The median age of the calves (n=107 from 58 herds) at the time of collection in the spring of 2002 was 6 days (range, 1 to 120 days). The age of the dams for these calves ranged from 2 to13 years (median, 5 years). Fifty-seven percent of the calves were male, and healthy calves accounted for 91.2% (98/107) of the population sampled.

Samples were also examined in the spring of 2002 from healthy cows (n=50) in 18 herds that ranged in age from 2 to 10 years (median, 5 years).

The remaining isolates were from calves (n=50 from 23 herds) sampled in the fall of 2002 for which the median age was 204 days (range, 118 to 301 days). Median dam age for these calves was 6 years (range, 2 to 14 years). Fifty-six percent of these calves were male and all calves were classified as healthy.

7.3.2. Phenotypic antimicrobial susceptibility in the selected isolates

Resistance to at least one antimicrobial was detected in 64.7% of the 207 selected isolates (Figure 7.2). The most common antimicrobials to which resistance was detected were tetracycline, sulphamethoxazole, and streptomycin (Table 7.3). No isolates were resistant to amikacin, ceftriaxone, ciprofloxacin, or nalidixic acid.

Twenty-nine different multiple resistance patterns were detected including the A3C, ACSSuT, AKSSuT, and ACKSSuT resistance patterns (Table 7.3). The most common pattern (17.9%, 37/207) contained a grouping of streptomycin, sulphamethoxazole, and

tetracycline. The next most common AMR pattern was sulphamethoxazole and tetracycline (10.6%, 22/207). The median number of antimicrobials observed per pattern was 3 and the maximum was 11.

7.3.3. Resistance genes detected in the selected isolates

Resistance genes were detected in 64.3% of all isolates tested, in 93.3 % (125/134) of resistant isolates, and in11.0 % (8/73) of susceptible isolates. Those most commonly detected included a gene for sulphonamide resistance, *sul*II, a gene for tetracycline resistance, *tet*B, and a gene for streptomycin resistance, *ant*(3")-Ia (*aad*A1) (Table 7.3). The resistance genes bla_{SHV}, ant(2")-Ia, *aac*(3)-IIa, *aph*(3")-IIa, and *dhfr*XV were not detected in any isolates.

Sixteen different multiple resistance gene patterns (≥2 resistance genes) were identified. The median number of resistance genes in the observed patterns was 2 with a maximum of 7. The most common pattern detected was *sul*II and *tet*B together (n=40, 19.3%). The next most common pattern (6 isolates) contained a grouping of bla_{TEM}, *aph*(3')-Ia, *tet*B, and *sul*II (2.9%).

Several different combinations of resistance genes comprising the ACSSuT, AKSSuT, or ACKSSuT patterns were identified. For example, 3 different resistance gene patterns in 6 isolates were identified for ACSSuT. AKSSuT isolates had 2 distinct patterns in 6 isolates. ACKSSuT isolates had 4 patterns in 7 isolates. Three of 5 isolates with the ACSSuT gene pattern had $bla_{TEM,}$ *cat*I, *ant*(3")-Ia (aadA1), *tet*A, *dhfr*I, and

*sul*I. Five of 6 isolates with the AKSSuT pattern had the *bla*$_{TEM,}$ *aph*(3')-Ia, *ant*(3")-Ia (*aad*A1), *sul*I, *tet*B, and *dhfr*I. The resistance genes detected in 4 of 7 isolates with the ACKSSuT pattern were *bla*$_{TEM,}$ *cat*I, *aph*(3')-Ia, *ant*(3")-Ia (*aad*A1), *tet*B, *dhfr*I, and *sul*I.

7.3.4. Phenotypic antimicrobial susceptibility and associated resistance genes

Genotypes did not always correspond with the phenotypic expression within individual isolates. Eight isolates carried resistance genes, but had no evidence of phenotypic resistance (Table 7.4). Alternatively there were also 8 isolates that were classified as resistant based on MICs with no resistance genes identified (Table 7.4). Genotypes did not correspond for 25.0% (2/8) of the ACSSuT, for 58.3% (7/12) of the AKSSuT, and for 33.3% (3/9) of the ACKSSuT phenotypes (Table7.5).

There were 122 isolates classified as resistant to tetracycline based on the MICs, of these isolates 15 (12%) were *tet*A, 83 (68%) were *tet*B, 4 (3.3%) were *tet*C, 3 (2.5%) were *tet*A and *tet*B together, 7 (5.7%) were *tet*A and *tet*C together, and 1 (0.8%) isolate was *tet*A, B, and C positive. There were also 9 (7.4%) isolates that were classified as resistant to tetracycline that did not have a corresponding tetracycline resistance gene.

Of the 114 sulphamethoxazole resistant isolates, 9 (7.9%) were *sul*I, 76 (67%) were *sul*II, 20 (18%) were *sul*I and *sul*II together, and 9 (7.9%) were positive for sulphamethoxazole phenotypically but not genotypically. For trimethoprim-sulphamethoxazole resistant isolates (n=33), 27 (81.8%) were *dhfr*I, 1 (3.0%) was

*dhfr*VII, 2 (6.1%) were *dhfr*XII, 1 (3.0%) was *dhfr*XII and *dhfr*XIII together, and 2 (6.1%) were *dhfr*Ib and *dfhr*V together. Sixty-seven (58.8%) of the 114 sulphamethoxazole resistant isolates were positive for at least 1 sulphonamide gene, 5 (4.4%) were positive for at least 1 sulphonamide gene and 1 trimethoprim gene, and 33 (28.9%) were positive for at least 1 sulphonamide gene, 1 trimethoprim gene, and were also phenotypically positive for trimethoprim-sulphamethoxazole.

The majority of the chloramphenicol resistant isolates (n=30) were *cat*I (n=21, 70.0%) positive, 5 (16.7%) were *flo*R positive, and 4 (13.3%) had no corresponding chloramphenicol resistance gene.

The gentamicin resistant isolates (n=2) had resistance genes *aac*(3)-IV and *ant*(3')-Ia(aada1) together (n=1) and *ant*(3')-Ia(aadA1) and *aph*(3')-Ia together (n=1). The 31 kanamycin resistant isolates had 14 (45.2%) with the *aph*(3')-Ia resistance gene, 1 (3.2%) with *ant*(3')-Ia(aadA1), 15 (48.3%) with *ant*(3')-Ia(aadA1) and *aph*(3')-Ia together, and 1 (3.2%) with no associated resistance gene. Almost half of the streptomycin resistant isolates (n=41, 47.7%) did not have an associated streptomycin resistance gene, 14 (16.3%) had *aph*(3')-Ia resistance gene, 9 (10.5%) had had *ant*(3')-Ia(aadA1), 20 (23.3%) had *ant*(3')-Ia(aadA1) and *aph*(3')-Ia together, 1 (1.1%) isolate had *ant*(3')-Ia(aadA1) and *ant*(3')-Ia(aadA6), and 1 (1.1%) had *aac*(3)-IV and *ant*(3')-Ia(aadA1) together.

7.3.5. Unconditional association between phenotypic antimicrobial susceptibility and identification of resistance genes

Phenotypic resistance to a number of antimicrobials was associated with the presence of the aminoglycoside genes *ant(3")*-Ia (*aadA1*) and *aph(3')*-Ia (Table 7.6). Positive associations were detected between phenotypes streptomycin and kanamycin and their respective resistance genes, *ant(3")*-Ia (*aadA1*) and *aph(3')*-Ia . Isolates with phenotypic ACKSSuT resistance were more likely to contain both the *aadA*1 and *aph*(3')-Ia genes than isolates without this pattern (Table 7.6).

Fewer unconditional associations were identified for the tetracycline genes than for the aminoglycoside genes (Table 7.7). Tetracycline resistance was not associated with the presence of the *tetA* gene, but was strongly associated with *tet*B.

Chloramphenicol resistant isolates were 63 and 18 times more likely to be either *cat*I gene or *flo*R gene positive, respectively, than chloramphenicol susceptible isolates (Table 7.8). Several of the extended spectrum cephalosporins were associated with *flo*R, but not with *cat*I. Significant associations between other phenotypic resistance patterns of interest and these genes were also detected.

Sulphamethoxazole resistance was associated with both *sul*I and *sul*II, but the association was much stronger for *sul*II (Table 7.9). Tetracycline resistance was strongly associated with both sulphonamide resistance genes. ACSSuT phenotype pattern was associated with *sul*I but not *sul*II.

Trimethoprim-sulphamethoxazole resistant isolates were associated with *dhfr*I, but not with *dhfr*XII (Table 7.10). Chloramphenicol resistant isolates were also associated with *dhfr*I, but not with *dhfr*XII. Associations with phenotypic patterns including ACSSuT, ACKSSuT, and A3C were also detected

Ampicillin resistant isolates were 86 times more likely to be positive for bla_{TEM} than ampicillin susceptible isolates (Table 7.11). ACSSuT and ACKSSuT resistant isolates were also more likely to be positive for bla_{TEM} than isolates susceptible to the ACSSuT and ACKSSuT patterns of antimicrobials.

Table 7.12 and Figure 7.3 summarize the associations between resistant phenotypes and the 6 antimicrobial families representing the 24 resistance genes investigated. The aminogylcoside gene *ant(3")*-Ia (*aadA1*) and the trimethoprim gene *dhfr*I were associated with every antimicrobial investigated. Resistance to ampicillin, kanamycin, streptomycin, sulphamethoxazole, and the ACSSuT pattern were associated with all 6 families of resistance genes investigated. Tetracycline and trimethoprim resistance were associated with 5 of the families while chloramphenicol and the ACKSSuT pattern were associated with 4 of the 6 families.

Isolates resistant to ≥1 antimicrobial were 74.3 (95% CI, 15.8-349, $P<0.0001$) times more likely to be positive for ≥1 of the resistance genes investigated than were susceptible isolates. Additionally, isolates resistant to ≥2 antimicrobials were 140 (95%

CI, 48.5-407, $P<0.0001$) times more likely to be positive for ≥2 resistance genes than isolates that were not resistant ≥2 antimicrobials. There was a increase of 1.2 (95% CI, 1.2-1.3, $P<0.0001$) resistance genes identified for every additional antimicrobial to which an isolate was resistant.

7.4. Discussion

The objective of this study was to measure the associations between AMR phenotypes and resistance genes in fecal generic *E. coli* isolates obtained from a study of cow-calf herds. To the best of our knowledge this study provides some of the first available information describing AMR both phenotypically and genotypically in cow-calf isolates. Additionally, it took a novel approach to investigating statistical associations between AMR phenotype and genotype. These data demonstrate extensive associations between various phenotypes and unrelated resistance genes. They illustrate the complex nature of AMR and would be useful in targeting future research projects.

Phenotypic resistance, as measured by microbroth dilution, was associated with the presence of at least one of the associated resistance genes for that antimicrobial. The relationship between isolates resistant to specific antimicrobials and related resistance genes was not unexpected, since a strong correlation between the phenotypic resistance pattern of a strain and the presence of resistance genes has previously been observed (van de Klundert et al., 1984, Shaw et al., 1991). In situations where a very strong association was detected between certain phenotypes and genotypes, the phenotypic expression of resistance to a particular antimicrobial may be a good indicator of the

underlying resistance gene for that antimicrobial especially in cases where the majority of resistance to those antimicrobials is caused by a single gene. Examples of such isolates from this population would include kanamycin and *aph*(3')-Ia, chloramphenicol and *cat*I, sulphamethoxazole and *sul*II, trimethoprim-sulphamethoxazole and *dhfr*I, and ampicillin and bla_{TEM}.

On the other hand, there was a lack of association between tetracycline resistance and the *tet*A resistance gene. This lack of association is probably because *tet*A is less likely to be found in isolates positive for *tet*B and most of the tetracycline resistant isolates were *tet*B positive. Jones et al. (1992) suggested an incompatibility of plasmids carrying the tetracycline resistance determinants could explain the existence of the negative associations between *tet*A and *tet*B. There was also no association between trimethoprim-sulphamethoxazole and the *dhfr*XII resistance gene. Trimethoprim dihydrofolate reductase (*dhfr*) belongs to the *dhfr* protein family that includes the chromosomally encoded trimethoprim sensitive and resistant *dhfr* genes of bacteria (Hall and Collis, 1998). It has been found that *dhfr*XII and *dhfr*XIII are closely related to each other but not to other members of the subgroup (Hall and Collis, 1998). Since the majority of the trimethoprim-sulphamethoxazole isolates were positive to *dhfr*I, the lack of relatedness between *dhfr*I and *dhfr*XII may explain why *dhfr*XII was not associated with trimethoprim-sulphamethoxazole resistance.

The associations between cefoxitin, ceftiofur, cephalothin and A3C, and the *flo*R resistance gene may indicate some degree of gene linkage. If linkage is present and

there is selection for florfenicol resistance, there may also be selection for resistance to these other antimicrobials or at least for the presence of these resistance genes. Because extended spectrum cephalosporins are important in human medicine, anything perpetuating the presence of these resistance genes is undesirable. Florfenicol resistance is typically located on a large transferable plasmid (Meuner et al., 2003), but it can also reside on chromosomes or non-conjugative plasmids (Singer et al., 2000). If *flo*R is being carried on a conjugative plasmid in these cow-calf isolates, the selection of the *flo*R gene may ultimately result in co-selection of other resistance genes in the presence of florfenicol since plasmids often carry multiple resistance genes (Cloeckaert et al., 2000). Thus, if resistance genes for extended-spectrum cephalosporins (ESCs) are linked to *floR* on such plasmids, ESC-resistance may be selected for by florfenicol use in cattle, even in the absence of ceftiofur use. This observation may therefore have important public health implications. Such plasmids have been found in porcine *E. coli* in Ontario (Travis et al., 2006). The MIC panel used in this study did not include florfenicol. Because florfenicol is approved for use in cattle in Canada and the presence of the *flo*R gene was detected, the frequency of florfenicol resistance requires further investigation.

For every additional antimicrobial to which phenotypic resistance was observed, another 1.2 resistance genes were detected. Phenotypic and genotypic characterization of the isolates demonstrated comparable patterns. Additionally, the most common drugs to which isolates demonstrated resistance were consistent with the most common resistance genes detected. Tetracycline, sulphamethoxazole, and streptomycin were the

top three drugs identified as being the most common for resistance measured either phenotypically or genotypically. The most commonly detected combinations of resistance phenotypes and resistance genes were also for sulphamethoxazole and tetracycline.

In many instances the phenotype or the genotype alone would not accurately predict the other. Molecular mechanisms underlying AMR are numerous and complex and the presence or absence of a specific gene corresponding to a particular phenotype does not necessarily imply that the particular strain is resistant or susceptible (Arrts, et al., 2006). DNA testing does not indicate whether an isolate is susceptible or resistant, but indicates if the gene is present or absent (Arrts et al 2006). Resistant phenotypes can emerge from many different genetic determinants and each determinant may present unique epidemiological features (Lanz et al., 2003). The divergence between genotype and phenotype could simply be explained by not testing for all possible resistance genes or by genes not being turned on within certain isolates. Examples of genes that were not tested for and that could account for the discrepancy between genotype and phenotype include *str*A/*str*B, *sul*III, *clm*A, and bla_{cmy2}. One other explanation for the difference in phenotypic resistance and the presence of resistance genes could be that the breakpoint may be misplaced resulting in misclassification of isolates as susceptible and resistance. Finally, some resistance phenotypes may be caused by point mutations rather than gene acquisition; therefore, no associated resistance gene would be expected. The genotypic and phenotypic polymorphism in this study was also described by Blake et al. (2003).

This study did not investigate the presence of class I integrons. Although an integron probe was not included, the ant(3")-1 (*aadA*1) probe was used. The *ant*(3")-Ia (*aadA1*) gene along with several different *dhfr* genes and the *sul*I gene have been located as gene cassettes within integrons (Lévesque et al., 1995, Fluit and Shmitz, 1999, Carattoli, 2001, Gestal et al., 2005). Despite not testing for integrons specifically, the strong associations along with the various patterns containing two or more of these genes may indicate the presence of integrons in this sample population.

A description of the extended spectrum beta-lactamases would also have been useful in this population. Read et al. (2005) found that antimicrobials commonly used in feedlot practices in western Canada could contribute to the presence of the $bla_{\text{CMY-2}}$ gene in generic *E. coli* isolated from these animals. The presences of these genes are particularly important as this class of antimicrobials is important in human medicine.

The A3C, ACSSuT, AKSSuT, and ACKSSuT resistance patterns were investigated for consistency with the reporting structure of the Canadian Integrated Program for Antimicrobial Resistance Surveillance or CIPARS (CIPARS, 2006). The spread of multi-drug resistant *Salmonella* Typhimurium DT104 (definitive type 104) by chromosomal integration of the genes encoding for resistance to ampicillin, chloramphenicol, streptomycin, sulphonamides, and tetracyclines (ACSSuT type) has been reported (Ihnot et al., 1998, Bolton et al., 1999, Casin et al., 1999, Schmieger and Schicklmaier, 1999). This is important because there is the ability for phylogenetically diverse gram negative clinical isolates to demonstrate similar patterns of resistance

(Rowe-Magnus, 2002). Also, since there is the ability for horizontal transfer of genetic material within and between microbial genera (Weldhagen, 2004) identifying analogous patterns to those of *S*. Typhimurium in commensal *E. coli* maybe relevant. Poppe et al. (2005) demonstrated the ability of resistance transfer between *E. coli* and *Salmonella*; therefore, the transfer of resistance from generic *E. coli* to zoonotic enteropathogens or vice versa is a concern. The relationship between the phenotype patterns and the resistance genes that make up these patterns indicate that there probably is an underlying molecular mechanism, such as plasmids or integrons that would explain the numerous associations between a phenotype and non-related resistance genes. To determine whether it is the same mechanism as found in *Salmonella* would require further molecular work.

For the phenotypes and resistance genes investigated the study did demonstrate that a phenotype does not necessarily reflect the underlying genotype and that a resistance gene does not always have an expressed phenotype. The associations between phenotype and underlying resistance genes were numerous and complex illustrating the likelihood of molecular linkage of resistance genes in this population. This is important since often only phenotype is reported. This information alone does not always provide a complete picture of what is happening at the molecular level. By assessing phenotype and genotype together researchers may examine if MIC breakpoints are appropriate, if there are emerging novel resistance genes responsible for a phenotype, if unrelated and un-expressed genes may impact future AMR emergence and if the current theories on prudent use are appropriate with the apparent extensive co-selection.

Both methods of characterization help to improve our understanding of the epidemiology of AMR. Assessment of AMR at the genetic level is an important tool in the understanding and the control of AMR (Lanz et al., 2003). It is apparent that the relationship between phenotypic resistance and the presence of resistance genes is extremely complicated. The extensive number of relationships between individual resistances or phenotypic resistance patterns and individual resistance genes or families of resistance genes suggests that there could be extensive linkage, and that there is probably co-selection when one type of resistance is being perpetuated. The type of linkages may be secondary to the recognition that AMR selection is not an independent process due to the complex nature of the associations between individual antimicrobials and resistance genes.

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7.6. References

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Figure 7.1. Schematic of number of samples, number of isolates and number of farms for each age group of animals investigated

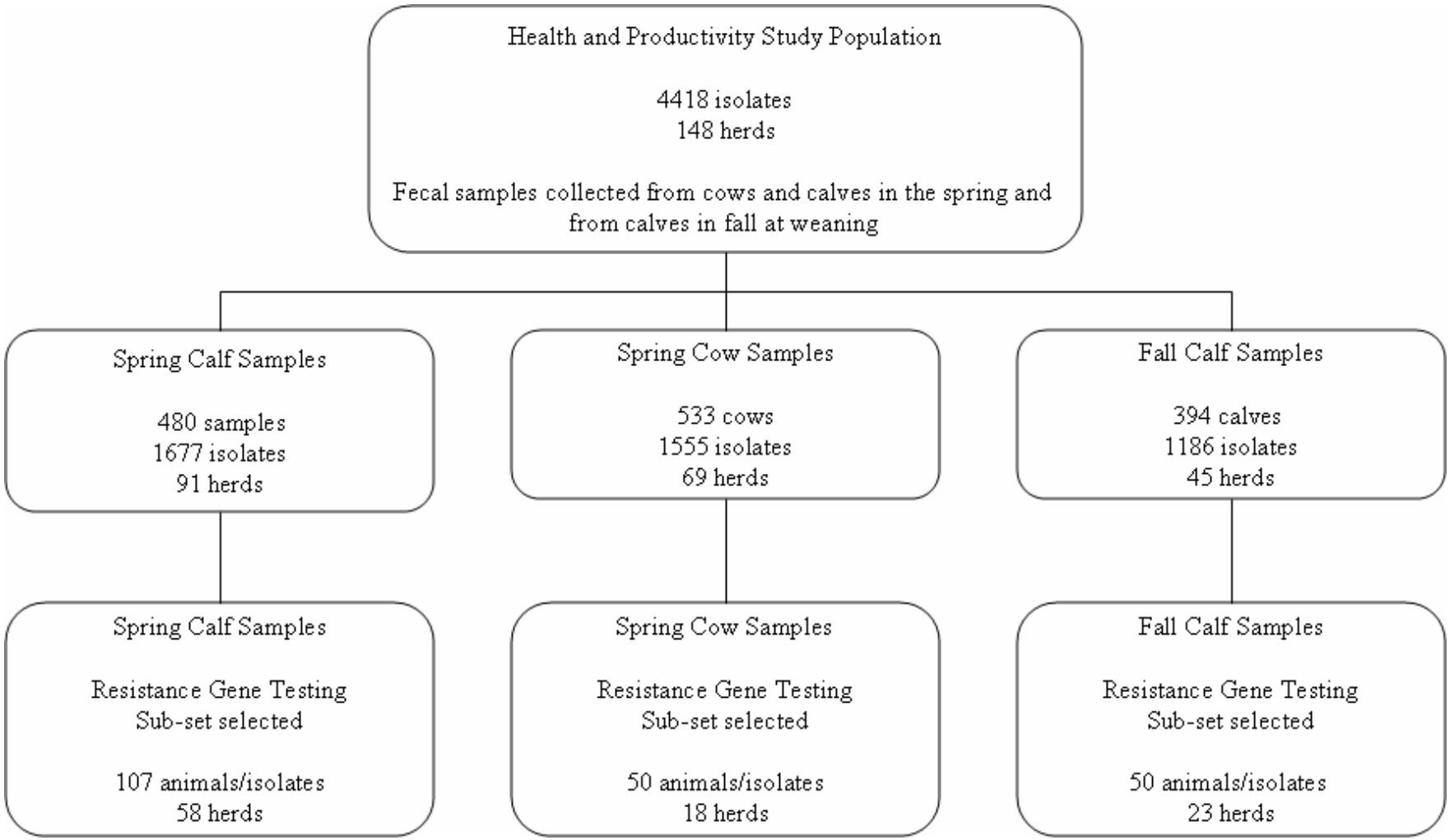
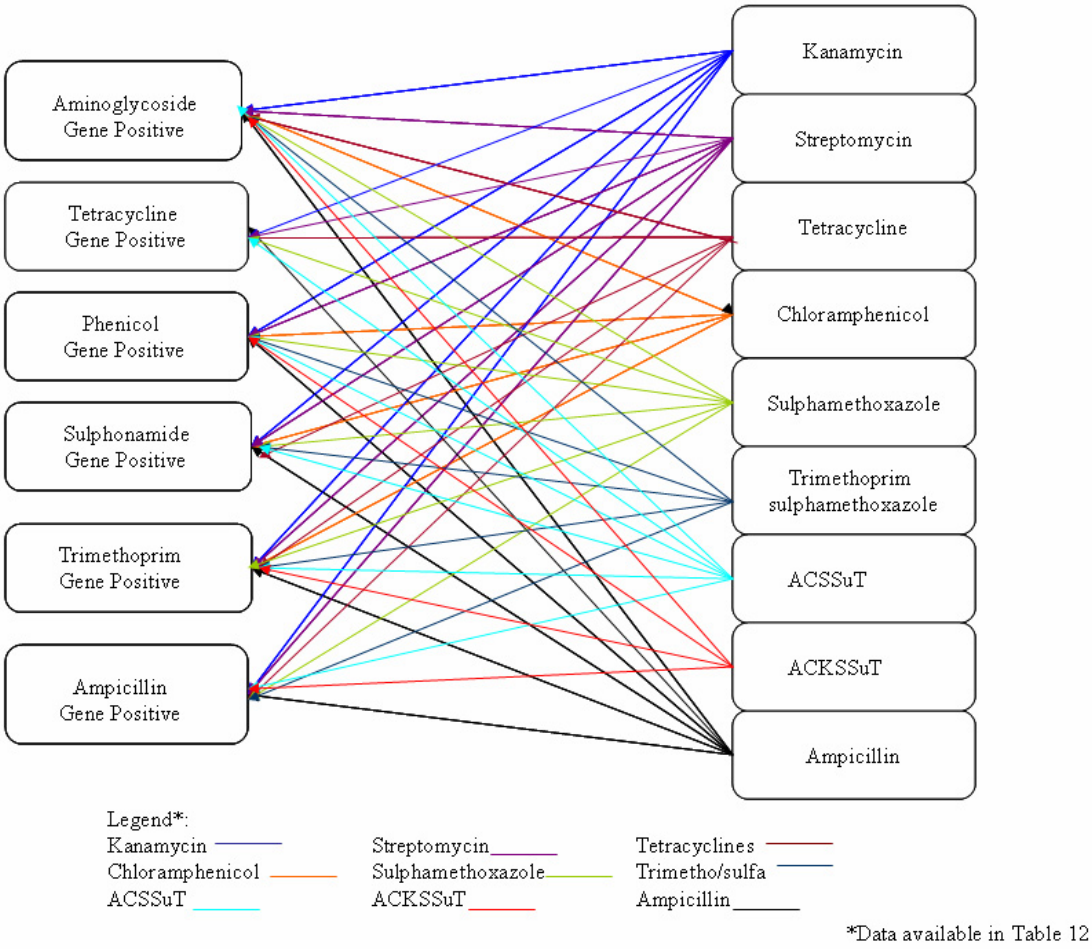


Figure 7.2. Minimum inhibitory concentration distribution for 207 isolates tested for antimicrobial susceptibility using Sensititre 2002 NARMS CMV7CNCD plate configuration. Numbers are presented as a percentage of the total isolates (n=207)

*	Antimicrobial	n	MIC Percentiles		Distribution of Isolates (%) Across Minimum Inhibitory Concentrations (MIC) Ranges															
			Median	75th	<=0.015	0.03	0.06	0.1	0.25	0.5	1	2	4	8	16	32	64	128	256	512	>512
I	Ceftiofur	207	0.25	0.25				2.4	72.9	20.8	1.0	0.5	1.0	1.0	**0.5**						
	Ceftriaxone	207	<=0.25	0.25					96.1	1.4			1.0	1.0	0.5						
	Ciprofloxacin	206	<=0.015	<=0.015	100.0																
II	Amikacin	207	2	2						0.5	33.8	61.4	3.9	**0.5**							
	Amoxicillin-Clavulanic Acid	207	4	4							0.5	25.6	53.6	12.6	2.9	2.4	**2.4**				
	Gentamicin	207	1	1					18.4	28.5	51.2	1.0			0.5	**0.5**					
	Kanamycin	207	<=8	<=8										85.0				**15.0**			
	Nalidixic Acid	207	4	4								42.5	57.5								
	Streptomycin	207	<=32	64												58.5	26.1	**15.5**			
	Trimethoprim-Sulphamethoxazole	207	0.25	0.5				49.8	19.8	14.0	0.5			**15.9**							
III	Ampicillin	207	4	4							3.4	35.7	39.6	2.4	0.5	0.5	**17.9**				
	Cefoxitin	207	4	4								15.0	64.7	15.0	0.5	**4.8**					
	Cephalothin	207	8	16									16.9	53.6	24.6	1.0	**3.9**				
	Chloramphenicol	207	4	8								3.9	46.9	33.8	1.0		**14.5**				
	Sulphamethoxazole	207	>512	>512											44.9					1.9	**53.1**
	Tetracycline	207	>=64	>=64									40.1	1.0	1.9	1.0	**56.0**				

Roman numerals I-IV indicate the ranking of human importance, Veterinary drug directorate, Health Canada. The un-shaded fields indicate the range tested for each antimicrobial in the 2002 plate configuration. Vertical double bars indicate the breakpoints used for categorization into susceptible and resistant. Numbers in bold font are the number of isolates with growth in all wells within the tested range, indicating the actual MIC is greater than that range of dilutions.

Figure 7.3. The complex nature of AMR phenotypes and families of resistance genes



*Data available in Table 12

Table 7.1. Antimicrobial family, genetic marker, primer sequence, GenBank accession number and DNA source for resistance genes tested

Antimicrobial family	Genetic marker	PCR primer sequence (5'-3')		Amplicon size (bp)	GenBank accession no.	Source of DNA
		Forward	Reverse			
Beta-lactams	*bla*$_{TEM}$	GAGTATTCAACATTTTCGT	ACCAATGCTTAATCAGTGA	857	AF309824	R. C. Levesque
	bla$_{SHV}$	TCGCCTGTGTATTATCTCCC	CGCAGATAAATCACCACAATG	768	AF148850	R. C. Levesque
Aminoglycosides	*aac(3)-IIa* (*aacC2*)	CGGAAGGCAATAACGGAG	TCGAACAGGTAGCACTGAG	740	X54723	D. Sandvang
	aac(3)-IV	GTGTGCTGCTGGTCCACAGC	AGTTGACCCAGGGCTGTCGC	627	X01385	J. Harel
	aph(3')-Ia (*aphA1*)	ATGGGCTCGCGATAATGTC	CTCACCGAGGCAGTTCCAT	600	M18329	J. Harel
	aph(3')-IIa (*aphA2*)	GAACAAGATGGATTGCACGC	GCTCTTCAGCAATATCACGG	680	V00618	J. Harel
	ant(3")-Ia(aadA1)	CATCATGAGGGAAGCGGTG	GACTACCTTGGTGATCTCG	786	DQ166553.1	J.Harel
	ant(3")-If (aadA6)	GAGTAACGCAGTACCCGC	CACTGGCATGGCACTAAGC	795	AY444814.1	J. Harel
Tetracycline	*tet*(A)	GTGAAACCCAACATACCCC	GAAGGCAAGCAGGATGTAG	888	X00006	J. Harel
	tet(B)	CCTTATCATGCCAGTCTTGC	ACTGCCGTTTTTTCGCC	774	J01830	J. Harel
	tet(C)	ACTTGGAGCCACTATCGAC	CTACAATCCATGCCAACCC	881	J01749	J. Harel
Phenicols	*catI*	AGTTGCTCAATGTACCTATAACC	TTGTAATTCATTAAGCATTCTGCC	547	M62822	J. Harel
	floR	CGCCGTCATTCCTCACCTTC	GATCACGGGCCACGCTGTGTC	215	AF252855	D. G. White
Trimethoprim	*dhfrI*	AAGAATGGAGTTATCGGGAATG	GGGTAAAAACTGGCCTAAAATTG	391	X00926	J. Harel
	dhfr1b	AGTATCATTGATAGCTGCG	GTAGTGCGCGAAGCGAAC	517	DQ388123.1	J. Harel
	dhfrV	CTGCAAAAGCGAAAAACGG	AGCAATAGTTAATGTTTGAGCTAAAG	432	X12868	O. Sköld
	dhfrVII	GGTAATGGCCCTGATATCCC	TGTAGATTTGACCGCCACC	265	X58425	O. Sköld
	dhfrIX	TCTAAACATGATTGTCGCTGTC	TTGTTTTCAGTAATGGTCGGG	462	X57730	C. Wallen
	dhfrXII	GAACTCGGAATCAGTACGC	ACGCGCATAAACGGAGTG	483	DQ157751.1	J. Harel
	dhfrXIII	CAGGTGAGCAGAAGATTTTT	CCTCAAAGGTTTGATGTACC	294	Z50802	P. V. Adrian
	dhfrXV	GGGAACAATTACTCTTC	GTCTTCAGATGATTTAGC	186	Z83311D	P. V. Adrian
Sulfonamides	*sulI*	TTCGGCATTCTGAATCTCAC	ATGATCTAACCCTCGGTCTC	822	X12869	R. C. Levesque
	sulII	CGGCATCGTCAACATAACC	GTGTGCGGATGAAGTCAG	722	M36657	J. Harel

Table 7.2. Investigation into the association between resistance phenotype and genotype included the following individual and groups of antimicrobials

Response variables	Risk factors for phenotype
bla$_{TEM}$	Ampicillin
aph(3')-Ia	Cefoxitin
*tet*A	Ceftiofur
*tet*B	Cephalothin
*tet*C	Chloramphenicol
catI	Kanamycin
floR	Streptomycin
*dhfr*I	Tetracycline
*dhfr*XII	Sulphamethoxazole
*sul*I	Trimethoprim/ Sulpha
*sul*II	
	A3C
	ACSSUT
	AKSSUT
	ACKSSUT

Table 7.3. Antimicrobial resistance phenotype and genotype prevalence (n=207)

Antimicrobial	Proportion % (# positive)	Antimicrobial	Resistance gene	Proportion % (# positive)
Amikacin	0.0	Ampicillin	*bla*$_{TEM}$	17.9 (37)
Amox/Clav.	4.8 (10)		*bla*$_{SHV}$	0.0
Ampicillin	18.4 (38)	Gentamicin	*aac*(3)-IV	0.5 (1)
Cefoxitin	4.8 (10)		*ant*(2")-Ia	0.0
Ceftiofur	1.5 (3)		*aac*(3)-Iia	0.0
Ceftriaxone	0.0	Neo/Kan[a]	*aph*(3')-Ia	17.9 (37)
Cephalothin	4.8(10)		*aph*(3")-IIa	0.0
Gentamicin	1.0 (2)	Strep/spectino[b]	*ant*(3")-Ia(*aadA1*)	19.3 (40)
Kanamycin	15.0 (31)		*ant*(3")_If (*aadA*6)	1.5 (3)
Streptomycin	41.6 (86)	Tetracycline	*tet*A	13.0 (27)
Chloramphenicol	14.5 (30)		*tet*B	45.4 (94)
Ciprofloxacin	0.0		*tet*C	8.7 (18)
Naladixic acid	0.0	Chloramphenicol	*cat*I	13.0 (27)
Sulphamethoxazole	55.1 (114)		*flo*R	3.4 (7)
Tetracycline	58.9 (122)	Trimethoprim	*dhfr*I	16.9 (35)
Trimethoprim/Sulpha	15.9 (33)		*dhfr*Ib	1.0 (2)
A3C	1.5 (3)		*dhfr*V	1.0 (2)
ACSSUT	4.4 (9)		*dhfr*VII	0.5(1)
AKSSUT	5.8 (12)		*dhfr*IX	0.5 (1)
ACKSSUT	3.9 (8)		*dhfr*XII	1.9 (4)
AMR +	64.7 (134)		*dhfr*XIII	0.5 (1)
			dhfrXV	0.0
		Sulphonamides	*sul*I	14.5 (30)
			*sul*II	48.3 (100)
		+ for any gene		64.3 (133)

[a]Neo/Kan=neomycin/kanamycin
[b]Strep/Spectino=streptomycin/spectinomycin

Table 7.4. Patterns of resistance genes present in isolates with susceptible phenotypes (n=8) and resistant phenotypes with no genotype (n=8)

Resistance Genes Present	# of isolates with this gene pattern and no phenotype	Phenotype
aph(3')-Ia / *tetB* / *sulII*	1	None
bla_{TEM}	1	None
bla_{TEM} / aph(3')-Ia / *ant(3")*Ia (*aadA1*)/ *dhfr*I / *dhfr*XII / *sulII*	1	None
bla_{TEM} / *tetB*	1	None
*dhfrI*X	1	None
tetA / *tetC*	1	None
tetC	2	None
Total number of isolates with no corresponding phenotype	8	

Phenotype Present	# of isolates with this phenotype and no corresponding resistance genes	Genotype
Sulphamethoxazole	6	None
Amoxicillin/clavulanic acid, ampicillin, cefoxitin	1	None
Streptomycin, tetracycline, sulphamethoxazole	1	None
Total number of isolates with no corresponding genotype	8	

Table 7.5. Table 12: Resistance genes detected for each of the ACSSuT, AKSSuT, and ACKSSuT phenotype patterns where the phenotype and genotype did not match

Phenotype Pattern	# of Isolates	Mismatched resistance genes for each phenotype pattern
ACSSuT (n=3)	1	*bla*$_{TEM}$,*aph*(3')-Ia, *ant*(3")-Ia (*aadA1*), *tet*B, *dhfr*I, *sul*II
	1	*tet*A, *tet*C, *flo*R, *sul*I
	1	*bla*$_{TEM,}$ *aph*(3')-Ia, *ant*(3")-Ia (*aadA1*), *tet*A, *flo*R, *dhfr*I, *dhfr*XII, *sul*I, *sul*II
AKSSuT (n=7)	5	*bla*$_{TEM}$,*aph*(3')-Ia, *tet*B, *sul*I
	2	*bla*$_{TEM,}$ *aph*(3')-Ia, *tet*A, *dhfr*Ib, *dhfr*V *sul*I, *sul*II
ACKSSUT (n=2)	1	*flo*R, *dhfr*I,*sul*II
	1	*aph*(3')-Ia, *ant*(3")-Ia (*aadA1*), *tet*A, *tet*B, *dhfr*XI, *sul*I

Table 7.6. Unconditional associations between being positive for individual or multiple AMR phenotypes and the antimicrobial gene *ant*(3")-Ia (*aadA1*) or *aph*(3')-Ia (n=207)

Response Variable	Risk Factor	OR	Confidence Interval		*P*-value
			Lower	Upper	
ant(3")-Ia (*aadA1*)	Ampicillin	9.4	4.2	21.2	0.0001
	Chloramphenicol	24.3[a]	9.9	59.3	0.0001
	Kanamycin	6.5	3.1	13.7	0.0001
	Streptomycin	6.6	2.8	15.2	0.0001
	Tetracycline	12.2	8.9	50.8	0.0006
	Sulphamethoxazole	23.5	18.8	113	0.0001
	Trimethoprim/sulpha	24.7	11.3	60.3	0.0001
	ACSSuT	8.2	2.3	30.0	0.0015
	ACKSSuT	30.6	3.2	291.4	0.0029
aph(3')-Ia	Ampicillin	12.2	4.1	36.2	0.0001
	Chloramphenicol	7.0	2.8	17.9	0.0001
	Kanamycin	306	64.9	1440	0.0001
	Streptomycin	26.4	8.1	86.1	0.0001
	Tetracycline	11.1	2.9	42.5	0.0004
	Sulphamethoxazole	12.7	3.8	42.7	0.0001
	Trimethoprim/sulpha	14.4	6.2	33.5	0.0001
	ACKSSuT	36.5	4.5	295.4	0.0007

Only statistically significant (P<0.004) associations are reported.
[a]*E*xample interpretation: isolates that are positive for chloramphenicol resistance are 24.3 (95% CI, 9.9-59.3, P=0.0001) times more likely to be positive to *aadA*1 than isolates that are chloramphenicol susceptible.

Table 7.7. Unconditional associations between being positive for various individual or multiple AMR phenotypes and the AMR genes *tet*A or *tet*B (n=207)

			Confidence Interval		
Response Variable	Risk Factor	OR	Lower	Upper	*P*-value
*tet*A	Ampicillin	6.1	1.9	19.4	0.0023
	Cefoxitin	4.9	1.7	13.8	0.0026
	ACSSuT	27.7	3.9	198.0	0.0009
*tet*B	Kanamycin	5.1	1.8	13.8	0.0017
	Streptomycin	7.3	3.5	15.3	0.0001
	Tetracycline	29.2	11.1	76.6	0.0001
	Sulphamethoxazole	18.1	8.0	40.8	0.0001

Only statistically significant ($P<0.004$) associations are reported.
No statistically significant associations for the *tet*C resistance gene

Table 7.8. Unconditional associations between being positive for various individual or multiple AMR phenotypes and the AMR genes *cat*I or *flo*R (n=207)

Response Variable	Risk Factor	OR	Confidence Interval		*P*-value
			Lower	Upper	
*cat*I	Ampicillin	5.5	2.1	14.3	0.0005
	Chloramphenicol	63.1	18.2	218.2	0.0001
	Kanamycin	8.1	3.1	21.5	0.0001
	Streptomycin	9.7	3.4	27.8	0.0001
	Sulphamethoxazole	26.2	3.0	224.7	0.0029
	Trimethoprim/sulpha	46.5	13.7	158.4	0.0001
	ACKSSuT	24.6	3.7	166.4	0.001
*flo*R	Cefoxitin	20.0	4.3	93.0	0.0001
	Ceftiofur	78.2	6.0	1021.6	0.0009
	Cephalothin	20.0	4.3	92.2	0.0001
	Chloramphenicol	17.5	3.5	87.4	0.0005
	A3C	78.2	6.0	1021.6	0.0009
	ACSSuT	24.0	5.3	108.1	0.0001

Only statistically significant ($P<0.004$) associations are reported.

Table 7.9. Unconditional associations between being positive for various individual or multiple AMR phenotypes and the AMR genes *sul*I or *sul*II (n=207)

Response Variable	Risk Factor	OR	Confidence Interval		*P*-value
			Lower	Upper	
*sul*I	Ampicillin	5.4	1.7	16.8	0.004
	Chloramphenicol	21.9	8.3	58.0	0.0001
	Kanamycin	6.3	2.0	20.1	0.0017
	Streptomycin	6.1	2.1	17.6	0.0009
	Tetracycline	26.2	2.9	235.6	0.0036
	Sulphamethoxazole	33.8	3.2	356.4	0.0034
	Trimethoprim/sulpha	20.3	6.6	62.6	0.0001
	ACSSuT	8.3	2.0	34.0	0.0034
*sul*II	Kanamycin	13.6	2.8	66.0	0.0012
	Streptomycin	18.7	7.1	49.4	0.0001
	Tetracycline	38.9	12.5	121.0	0.0001
	Sulphamethoxazole	125.4	41.5	378.4	0.0001

Only statistically significant ($P<0.004$) associations are reported.

Table 7.10. Unconditional associations between being positive for various individual or multiple AMR phenotypes and the AMR gene *dhfr*I or *dhfr*XII (n=207)

Response Variable	Risk Factor	OR	Confidence Interval		*P*-value
			Lower	Upper	
*dhfr*I	Ampicillin	9.0	4.0	19.9	0.0001
	Chloramphenicol	57.4	16.0	206.0	0.0001
	Kanamycin	11.9	5.1	27.8	0.0001
	Streptomycin	16.8	5.2	53.6	0.0001
	Tetracycline	8.7	2.1	35.9	0.0028
	Sulphamethoxazole	18.3	3.9	85.2	0.0002
	Trimethoprim/sulpha	121.3	30.6	480.7	0.0001
	ACSSuT	9.4	2.5	35.5	0.0009
	ACKSSuT	35.3	5.1	242.6	0.0003
*dhfr*XII	Ceftiofur	24.9	3.6	171.5	0.0011
	A3C	24.9	3.6	171.5	0.0011
	ACSSuT	24.3	4.1	144.7	0.0005

Only statistically significant ($P<0.004$) associations are reported.

Table 7.11.Unconditional associations between being positive for various individual or multiple AMR phenotypes and the AMR gene any *bla*$_{TEM}$ (n=207)

Response Variable	Risk Factor	OR	Confidence Interval		*P*-value
			Lower	Upper	
bla$_{TEM}$	Ampicillin	85.8	26.8	275.2	0.0001
	Kanamycin	17.5	5.9	51.9	0.0001
	Streptomycin	11.0	3.6	33.6	0.0001
	Tetracycline	7.6	2.4	24.4	0.0006
	Sulphamethoxazole	4.2	1.7	10.5	0.0019
	Trimethoprim/sulpha	9.9	3.8	25.9	0.0001
	ACSSuT	10.1	2.9	35.5	0.0003
	ACKSSuT	14.7	3.3	66.1	0.0005

Only statistically significant (P<0.004) associations are reported.

Table 7.12. Summary of associations between various antimicrobials and each family of resistance genes

Antimicrobial	Aminoglycoside gene +		Tetracycline gene +		Phenicol gene +		Sulphonamide gene +		Trimethoprim gene +		Ampicillin gene +
	ant(3'')-Ia (*aadA1*)	*aph(3')*-Ia	*tet*A	*tet*B	*cat*I	*flo*R	*sul*I	*sul*II	*dhfr*I	*dhfr*XII	bla_{TEM}
Ampicillin	+	+	+	N	+	N	+	N	+	N	+
Chloramphenicol	+	+	N	N	+	+	+	N	+	N	N
Kanamycin	+	+	N	+	+	N	+	+	+	N	+
Streptomycin	+	+	N	+	+	N	+	+	+	N	+
Tetracycline	+	+	N	+	N	N	+	+	+	N	+
Sulphamethoxazole	+	+	N	+	+	N	+	+	+	N	+
Trimethoprim Sulphamethoxazole	+	+	N	N	+	N	+	N	+	N	+
ACKSSuT	+	+	N	N	+	N	N	N	+	N	+
ACSSuT	+	N	+	N	N	+	+	N	+	+	+

+ association between the resistance gene and the phenotype
N= no association between the resistance gene and the phenotype
For more details about the above associations please refer to Tables 5 through 11

CHAPTER 8
MOLECULAR CHARACTERIZATION OF AMR IN FECAL GENERIC *ESCHERICHIA COLI* ISOLATES FROM WESTERN CANADIAN COW-CALF HERDS: PART II ASSOCIATIONS BETWEEN RESISTANCE GENES

8.1. Introduction

Antimicrobial resistance (AMR) is an important issue in both human and veterinary medicine. Many studies have focused on organisms that are pathogenic for people including; *Salmonella* spp. (Wray et al., 1991), *Campylobacter* spp. (Gaunt and Piddock, 1996), or *Escherichia coli* O157 (Meng et al., 1998). Transmissible genetic elements encoding AMR can also be maintained in commensal bacteria (Shaw and Cabelli, 1980, Salyers and Shoemaker, 1996, Falagas and Siakavelllas, 2000). Resistance gene transmission from nonpathogenic to pathogenic organisms within the intestinal tract may be important for the development of AMR (Winokur et al., 2001). *Escherichia coli* have developed a number of elaborate methods for acquiring and disseminating genetic determinants and may serve as a reservoir for transmissible resistance (Neidhardt, 1996). Studying the molecular determinants of AMR in generic *E. coli* will increase our understanding of the significance of these bacteria in the development and transfer of AMR.

Antimicrobial use (AMU) has an impact on the distribution of AMR phenotypes (McGowan and Gerding, 1996, Gaynes and Monnet, 1997, Aarestrup. 1999) and

resistance gene possession (Blake et al., 2003). As a result of varying AMU practices in different livestock species, describing AMR in one livestock class may not be representative of another. To date there is little information available on AMR in cow-calf herds and no data for western Canada, although the western provinces of Alberta and Saskatchewan contain more than 65% of the beef cow, breeding heifer and calf populations in Canada (Statistics Canada, Accessed July 25, 2006; http://www.40.statcan.ca/101/cst01/prim50a.htm). Describing AMR throughout all phases of livestock production is vital to understanding the epidemiology of AMR.

The spread of mobile genetic elements such as plasmids, transposons, or integron/gene cassettes (Hall and Collins, 1995, Bennett, 1999, Schwarz and Chaslus-Dancla, 2001) may be responsible for the rapid dissemination multiple AMR genes (Kruse and Sorun, 1994, Salyers and Cuevas, 1997, Sandvang et al., 1997). Linked clusters of AMR on a single mobile element can also aggregate in such a way that antimicrobials of a different class or even non-antimicrobial substances like heavy metals or disinfectants can select for AMR bacteria (Recchia and Hall, 1997, Salyers et al., 2004). Exchange of resistance genes between pathogens and non-pathogens or between gram positive and negative bacteria has also been documented (Prescott, 2000, Salyers et al., 2004).

Co-selection of resistance genes has a substantial impact on the implementation of prudent antimicrobial use guidelines. Since resistance genes can be linked on mobile genetic elements use of a particular antimicrobial can select for resistance not only to

that antimicrobial but potentially to a variety of others. This means that even if there is restricted use of certain antimicrobials, the resistance genes associated with these restricted antimicrobials could still be perpetuated through co-selection. By understanding the associations between resistance genes the impact of certain prudent use guidelines can be considered. The objective of this study was to explore associations between genetic determinants of AMR in fecal generic *E. coli* isolates obtained from cow-calf herds in western Canada.

8.2. Materials and methods

As a part of a strategic research initiative to study AMR and AMU in cow-calf herds (Gow and Waldner, 2007, Gow et al., 2007a,b,c), this project was undertaken to compliment a project examining associations between AMR phenotype and resistance genes (Gow et al., 2007c). The focus of this paper is on associations between resistance genes to explore the potential for co-selection of AMR genetic determinants in commensal *E. coli*.

Materials and methods have been described in detail elsewhere (Gow et al., 2007c). Briefly, the 207 isolates utilized for this study were a selected sub-set from a larger study investigating AMR in cow-calf herds. The isolates were divided into susceptible or resistant. Since genetic determinants of AMR were of interest the majority of isolates (65%, 134/207) selected for this project were classified as resistant phenotypically. From either the susceptible or resistant list, isolates were randomly selected ensuring that not ≥1isolate from the same animal was included. Fecal samples

were cultured for generic *E. coli.* A minimum of three isolates per sample identified as *E. coli* were selected and stored at -80°C until susceptibility testing could be performed. Isolates were tested for susceptibility using a microbroth dilution technique (Sensititre®, TREK Diagnostic Systems Inc., Cleveland, Ohio) and the standard 2002 National Antimicrobial Resistance Monitoring System (NARMS) public health panel (CIPARS, 2006). All testing was done in accordance with National Committee on Clinical Laboratory Standards (NCCLS) guidelines (NCCLS, 2000). DNA hybridization and PCR were used to test for 24 resistance genes from 6 antimicrobial families was done on the 207 isolates (Table 8.1). Details on isolate phenotype have been described elsewhere (Gow et al., 2007c).

8.2.1. Statistical analysis

Descriptive analyses were completed and variables were recoded as necessary for statistical modeling using commercially available software programs (SPSS 11.0 for Windows, SPSS Inc., Chicago, Illinois). Initially all isolates were coded as to the presence or absence of each gene considered in the analysis. Isolates were further categorized for the presence or absence of at least one gene for each of the six families of antimicrobials considered in this study. For example; if an isolate contained any individual *tet* resistance gene or any combination of *tet* resistance genes it would have been classified as being positive for the appropriate individual genes, but it also would have been classified as being tetracycline gene positive as well. Mulitple resistance was defined as an isolate with the presence of ≥2 resistance genes.

Outcome and response variables of interest included the individual resistance genes with a prevalence of greater than 1.5% that could contribute to specific AMR patterns as well as the resistance genes *dhfr*I and *dhfr*XII (Table 8.2). Generalized estimating equations (GEE) were used to account for clustering of isolates sampled within herd (SAS v.8.2 for Windows (PROC GENMOD); SAS Institute, Cary, North Carolina, USA). Model specifications included a binomial distribution, logit link function, repeated statement with subject equal to herd, and an exchangeable correlation structure.

Statistically significant associations were reported as odds ratios (OR) with 95% confidence limits. Odds ratios >1 indicate an increasing occurrence of the genotype being studied with the other genotype being measured (a positive association) while those <1 indicate a decreasing occurrence of the genotype being studied with increases in the other measured genotype (a negative association). Multiple comparisons were accounted for using a Bonferroni correction to provide a conservative estimate for the level of statistical significance (Dohoo et al., 2003) An association was significant if $P<0.004$ after correction for 12 comparisons ($P < 0.05/k$, k = number of comparisons) (Dohoo et al., 2003).

8.3. Results

8.3.1. Description of sample population for genotyping study

In the spring of 2002, samples (n=107) were collected from calves with a median age of 6 days and ranging in age from of 1 to 120 days. Dam age for these calves ranged from 2 to13 years with a median cow age of 5 years. Fifty-seven percent of the calves were male, and calves not currently showing clinical signs of disease accounted for 91.2% (98/107) of the population sampled.

Samples (n=50) were also collected from healthy cows that ranged in age from 2 to 10 years with a median age of 5 years.

The median age for calves sampled in the fall (n=50) was 204 days with a range of 118 to 301. Median dam age for these calves was 6 years (range, 2 to 14 years). Fifty-six percent of these calves were male and all calves were classified as healthy.

8.3.2. Resistance genes detected in the selected isolates

Resistance genes were detected in 64.3% of the isolates. The most common resistance genes detected included a gene for sulphonamide resistance, *sul*II, a gene for tetracycline resistance, *tet*B, and a gene for streptomycin resistance, ant(3")-Ia (aadA1) (Table 8.3). No isolates carried the resistance genes $bla_{SHV,}$ *ant*(2")-Ia, *aac*(3)-IIa, *aph*(3")-IIa, and *dhfr*XV . Low levels of *aac*(3)-IV, *ant*(3")-If (*aad*A6) and several resistance genes for trimethoprim were detected.

Sixteen different multiple resistance gene patterns were identified in 106 of the isolates. For the multi-resistance gene isolates, the median number of resistance genes was 2 with a maximum of 7. The most common pattern detected included *sul*II and *tet*B together (n=40, 19.8%). The next most common pattern (6 isolates) contained a grouping of bla_{TEM}, *aph*(3')-Ia, *tet*B and *sul*II (2.9%).

8.3.3. Unconditional association between resistance genes

Numerous associations were detected between the various resistance genes examined and the aminoglycoside genes (Table 8.4). At least one resistance gene in each family of antimicrobials considered was associated with the aminoglycoside gene *ant*(3")-Ia (*aad*A1 (Table 4). Both *ant*(3")-Ia (*aad*A1 and *aph*(3')-Ia were also strongly associated with each other.

Fewer associations were identified among the various resistance genes examined and the tetracycline genes (Table 8.5) than for the aminoglycoside genes. *Tet*A and *tet*C were significantly associated and were often found in the same isolate. However, *tet*A positive isolates were less likely to be *tet*B positive than isolates that were *tet*A negative (Table 8.5). There was no significant association between *tet*B and tetC.

Phenicol genes *cat*I and *flo*R were not associated with each other. Two different resistance genes from the trimethoprim family had the strongest associations with either the *cat*1 or the *flo*R genes (Table 8.6).

Of the resistance genes tested, chloramphenicol, tetracycline and streptomycin genes were the most strongly associated with sulphonamide genes (Table 8.7). Resistance genes *sul*I and *sulI*II were not associated with each other.

Very strong associations were also detected between the phenicol resistance genes and the trimethoprim resistance genes (Table 8.8). Neither trimethoprim gene was associated with the other.

Numerous associations between individual genes and bla_{TEM} resistance genes were detected with the strongest association between (aph3')-Ia and bla_{TEM} (Table 8.9).

Table 8.10 and Figure 8.1 summarize the associations between individual resistance genes at the antimicrobial family level. At least one resistance gene from every other family of antimicrobials was significantly associated with at least one resistance gene belonging to the aminoglycoside family.

8.4. Discussion

To gain a better understanding of the epidemiology and the implications of AMR in cow-calf herds, fecal generic *E. coli* isolates obtained from cow-calf herds were examined to describe the associations between resistance genes. The complex nature of AMR was demonstrated by the large number of associations of moderate to substantial magnitude that were detected between resistance genes.

Resistance genes are often associated with integrons or mobile DNA such as plasmids and transposons that facilitate resistance gene distribution (Jacoby, 1994, Tenover and Rasheed, 1998). The presence of resistance genes on plasmids or on bacterial chromosomes suggests that genes conferring multi-drug resistance can exist as complex configurations of physically linked elements (Carattoli, 2001). The large number of strong associations between genes is consistent with the hypothesis that there is linkage between many of these resistance genes. The exact mechanism of linkage cannot be determined by the current study, but further molecular investigation would demonstrate potential gene linkages and the location of gene clusters on mobile genetic elements.

In addition to resistance acquisition, some of resistance genes and associations between resistance genes might be accounted for by common AMU on cow-calf farms. Direct use of antimicrobials can drive the co-selection of resistance genes. An example of this phenomenon was described by O'Conner et al., (2002) who determined that the use of injectable oxytetracycline in cattle receiving chlortetracycline in their feed was associated with an increase in the incidence of resistance to chloramphenicol and sulphasoxazole. Similar mechanisms might underlie the patterns demonstrated in these cow and calf isolates since different patterns of co-selection can be dependent on AMU patterns (Lanz et al, 2003).

Antimicrobials commonly used in western Canadian cow-calf herds are tetracyclines, sulphonamides and trimethoprim/sulphonamides (Gow and Waldner,

2007). These antimicrobials are consistent with some of the most prevalent resistance genes demonstrated in this study. However, despite the ban of chloramphenicol use in food producing animals since 1985 (Gilmore, 1986), chloramphenicol resistance was expressed by these isolates. This may indicate that co-selection is contributing to the persistence of chloramphenicol resistance genes in the population (Travis et al., 2006). In this study, the chloramphenicol gene *cat*I was associated with the presence of both a trimethoprim gene (*dhfr*I) and a sulphonamide gene (*sul*I). In beef cattle there may be co-selection for the chloramphenicol resistance gene resulting from selecting for trimethoprim or sulphamethoxazole resistance. Further investigation into the molecular relationship and the potential link to AMU is needed.

There was a negative association between *tet*A and *tet*B resistance genes. An incompatibility of plasmids carrying the tetracycline resistance determinants could explain the existence of the negative association between *tet*A and *tet*B (Jones et al., 1992). No association was detected between *sul*1 and *sul*II. Potentially the plasmid incompatability and the strong association of *tet*A with *sul*I and *tet*B with *sul*II could account for this lack of association. The absence of an association between *dhfr*I and *dhfr*XII was not unexpected because *dhfr*XII and *dhfr*XIII are closely related to each other but not to other members of the subgroup (Hall and Collis, 1998).

This study did not investigate the presence of class I integrons or extended-spectrum beta-lactamases. Although an integron probe was not included, the *ant*(3")-1 (*aadA*1) probe was used. The *ant*(3")-Ia (*aadA1*) gene, along with several different *dhfr* genes

and the *sul*I gene, have been located as gene cassettes within integrons (Lévesque et al., 1995, Fluit and Shmitz, 1999, Carattoli, 2001, Gestal et al., 2005). When examining associations between these three genes they were all strongly associated with each other as well as with resistance genes bla_{TEM}, *cat*1, *aph*(3')-Ia. Despite not testing specifically for integrons, the various patterns containing two or more of these genes suggested the presence of integrons in this sample population.

To understand the implication of the multiple associations detected between individual resistance genes, higher-level associations were examined between indicators of genetic resistance to families of antimicrobials. While the authors do recognize, particularly for the aminoglycoside family of antimicrobials, that resistance to one antimicrobial within this family does not confer resistance to the entire family (Salyers and Whitt, 2005), the goal of this investigation was simply to create an initial picture of complex nature of resistance between the families of antimicrobials used in cow-calf herds. This exploration provided further insight into the complexity of the epidemiology of AMR. This network of associations also brings into question the definition of "prudent use" and the impact of these associations on developing policy and clinical practice guidelines to minimize AMR. The implication is that current attempts to limit the emergence or spread of AMR based on careful restriction of the choice of antimicrobials will not prevent selection for a number of unrelated AMR genes. Therefore, the assessment of AMR at the genetic level is a critical tool in the understanding and the potential control of AMR (Lanz et al., 2003). This research

provides a baseline of important resistance gene relationships that need to be considered when planning potential AMR control in cow-calf herds.

8.5. Acknowledgements

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8.6. References

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Figure 8.1. Resistance gene relationships between antimicrobial families. Each line represents one of the relationships detailed in Tables 4 to 9

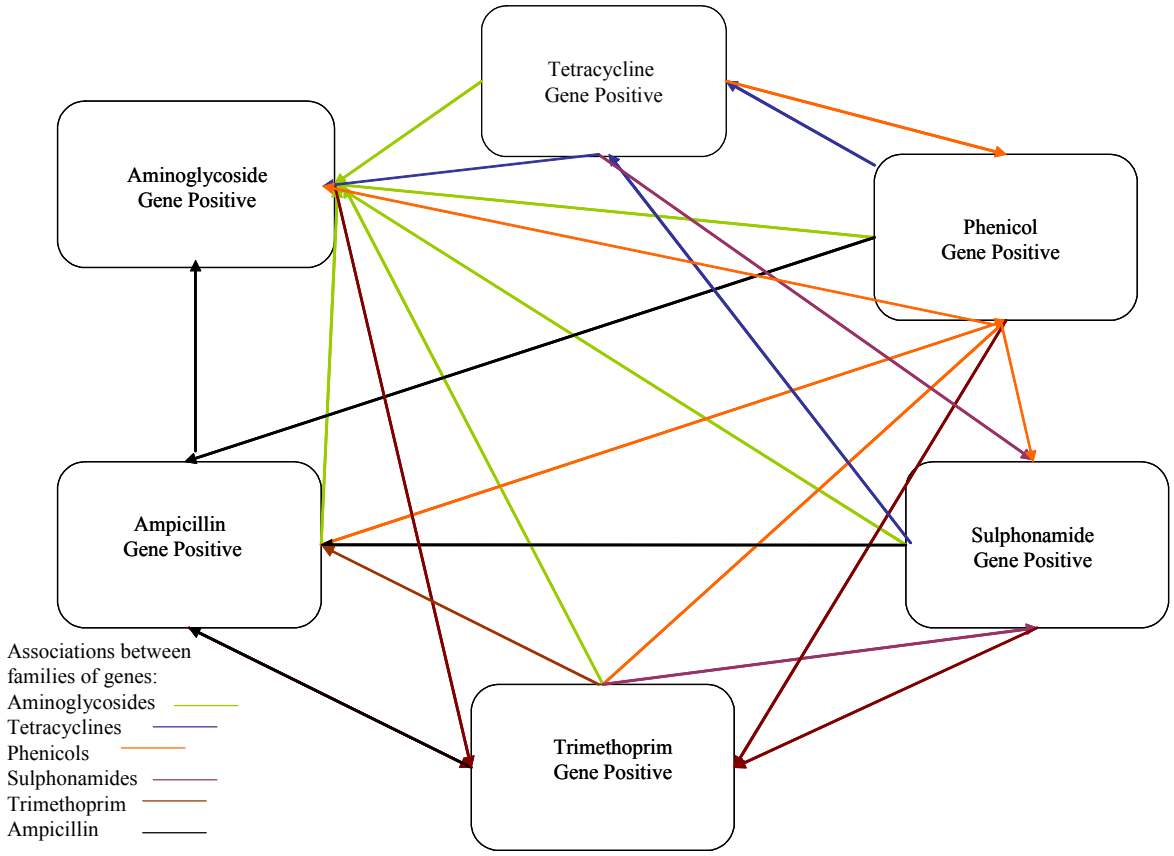

Table 8.1. Antimicrobial family, genetic marker, primer sequence, GenBank accession number and DNA source for resistance genes tested

Antimicrobial family	Genetic marker	PCR primer sequence (5'-3')		Amplicon size (bp)	GenBank accession no.	Source of DNA
		Forward	Reverse			
Beta-lactams	*bla*$_{TEM}$	GAGTATTCAACATTTTCGT	ACCAATGCTTAATCAGTGA	857	AF309824	R. C. Levesque
	bla$_{SHV}$	TCGCCTGTGTATTATCTCCC	CGCAGATAAATCACCACAATG	768	AF148850	R. C. Levesque
Aminoglycosides	*aac(3)-IIa* (*aacC2*)	CGGAAGGCAATAACGGAG	TCGAACAGGTAGCACTGAG	740	X54723	D. Sandvang
	aac(3)-IV	GTGTGCTGCTGGTCCACAGC	AGTTGACCCAGGGCTGTCGC	627	X01385	J. Harel
	aph(3')-Ia (*aphA1*)	ATGGGCTCGCGATAATGTC	CTCACCGAGGCAGTTCCAT	600	M18329	J. Harel
	aph(3')-IIa (*aphA2*)	GAACAAGATGGATTGCACGC	GCTCTTCAGCAATATCACGG	680	V00618	J. Harel
	ant(3")-Ia(aadAI)	CATCATGAGGGAAGCGGTG	GACTACCTTGGTGATCTCG	786	DQ166553.1	J.Harel
	ant(3")-If (aadA6)	GAGTAACGCAGTACCCGC	CACTGGCATGGCACTAAGC	795	AY444814.1	J. Harel
Tetracycline	*tet*(A)	GTGAAACCCAACATACCCC	GAAGGCAAGCAGGATGTAG	888	X00006	J. Harel
	tet(B)	CCTTATCATGCCAGTCTTGC	ACTGCCGTTTTTTCGCC	774	J01830	J. Harel
	tet(C)	ACTTGGAGCCACTATCGAC	CTACAATCCATGCCAACCC	881	J01749	J. Harel
Phenicols	*catI*	AGTTGCTCAATGTACCTATAACC	TTGTAATTCATTAAGCATTCTGCC	547	M62822	J. Harel
	floR	CGCCGTCATTCCTCACCTTC	GATCACGGGCCACGCTGTGTC	215	AF252855	D. G. White
Trimethoprim	*dhfrI*	AAGAATGGAGTTATCGGGAATG	GGGTAAAAACTGGCCTAAAATTG	391	X00926	J. Harel
	dhfr1b	AGTATCATTGATAGCTGCG	GTAGTGCGCGAAGCGAAC	517	DQ388123.1	J. Harel
	dhfrV	CTGCAAAAGCGAAAAACGG	AGCAATAGTTAATGTTTGAGCTAAAG	432	X12868	O. Sköld
	dhfrVII	GGTAATGGCCCTGATATCCC	TGTAGATTTGACCGCCACC	265	X58425	O. Sköld
	dhfrIX	TCTAAACATGATTGTCGCTGTC	TTGTTTTCAGTAATGGTCGGG	462	X57730	C. Wallen
	dhfrXII	GAACTCGGAATCAGTACGC	ACGCGCATAAACGGAGTG	483	DQ157751.1	J. Harel
	dhfrXIII	CAGGTGAGCAGAAGATTTTT	CCTCAAAGGTTTGATGTACC	294	Z50802	P. V. Adrian
	dhfrXV	GGGAACAATTACTCTTC	GTCTTCAGATGATTTAGC	186	Z83311D	P. V. Adrian
Sulfonamides	*sulI*	TTCGGCATTCTGAATCTCAC	ATGATCTAACCCTCGGTCTC	822	X12869	R. C. Levesque
	sulII	CGGCATCGTCAACATAACC	GTGTGCGGATGAAGTCAG	722	M36657	J. Harel

Table 8.2. Investigation into the association between genotypes included the following individual and groups of antimicrobials. Each gene in the response variable column was individually tested for associations with each gene in the risk factors column

Response variables	Risk factors for genotype
bla$_{TEM}$	*bla*$_{TEM}$
aph(3')-Ia	*aph*(3')-Ia
ant(3")-Ia	*Ant(3")*-Ia
*tet*A	*tet*A
*tet*B	*tet*B
*tet*C	*tet*C
catI	*catI*
floR	*floR*
*dhfr*I	*dhfr*I
*dhfr*XII	*dhfr*XII
*sul*I	*sul*I
sulII	*sulII*

Table 8.3. List of antimicrobial agents and the associated resistance genes investigated along with the resistance gene prevalence for 207 isolates from beef cattle

Antimicrobial	Resistance gene	Prevalence % (# Positive)
Ampicillin	*bla*$_{TEM}$	17.9 (37)
	bla$_{SHV}$	0.0
Gentamicin	*aac*(3)-IV	0.5 (1)
	ant(2")-Ia	0.0
	aac(3)-Iia	0.0
Neomycin/ Kanamycin	*aph*(3')-Ia	17.9 (37)
	aph(3")-IIa	0.0
Streptomycin/ Spectinomycin	*ant*(3")-Ia (*aadA1*)	19.3 (40)
	ant(3")_If (*aadA*6)	1.5 (3)
Tetracycline	*tet*A	13.0 (27)
	*tet*B	45.4 (94)
	*tet*C	8.7 (18)
Chloramphenicol	*cat*1	13.0 (27)
	*flo*R	3.4 (7)
Trimethoprim	*dhfr*I	16.9 (35)
	*dhfr*Ib	1.0 (2)
	*dhfr*V	1.0 (2)
	*dhfr*VII	0.5(1)
	*dhfr*IX	0.5 (1)
	*dhfr*XII	1.9 (4)
	*dhfr*XIII	0.5 (1)
	dhfrXV	0.0
Sulphonamides	*sul*I	14.5 (30)
	*sul*II	48.3 (100)
Gene for AMR Yes/No		64.3 (133)

Table 8.4. Unconditional associations detected between being positive for individual resistant genes and the antimicrobial gene *ant*(3")Ia (*aadA1*) and *aph*(3')-Ia (n=207)

Response Variable	Risk Factor	OR	95% Confidence Interval		*P*-value
			Lower	Upper	
ant(3")-Ia (*aadA1*)	bla_{TEM}	11.5	4.9	27.2	0.0001
	*cat*1	53.9	13.2	221	0.0001
	aph(3')-Ia	10.0	4.5	22.4	0.0001
	*tet*A	6.2	2.3	16.6	0.0003
	*su1*I	52.3	11.7	233	0.0001
	dhrfI	96.2[a]	30.2	306	0.0001
aph(3')-Ia	bla_{TEM}	22.7	6.6	78.1	0.0001
	*cat*1	9.0	3.5	22.7	0.0001
	ant(3")-Ia (*aadA1*)	10.7	4.7	24.5	0.0001
	*tet*B	16.8	5.0	56.8	0.0001
	*su1*I	5.9	2.3	15.1	0.0002
	*sul*II	17.4	4.2	71.6	0.0001
	dhfrI	18.2	7.1	47.2	0.0001

Only statistically significant ($P<0.004$) associations are reported

[a]*E*xample interpretation: isolates that are positive for the resistance gene *dhfr*I are 96.2 (95% CI, 30.2-306.3, $P=0.0001$) times more likely to be positive to *aadA1* than isolates that are *dhfr*I negative isolates.

Table 8.5. Unconditional associations detected between being positive for individual resistant genes and the antimicrobial resistance genes *tet*A, *tet*B or *tet*C (n=207)

Response Variable	Risk Factor	OR	95% Confidence Interval		*P*-value
			Lower	Upper	
*tet*A	*ant*(3")-Ia (*aadA1*)	6.7	2.5	17.9	0.0001
	*tet*B	0.2	0.1	0.5	0.0012
	*tet*C	8.7	2.4	30.7	0.0008
	*sul*I	15.0	5.6	40.0	0.0001
*tet*B	*aph*(3')-Ia	9.5	3.3	27.1	0.0001
	*tet*A	0.2	0.1	0.5	0.0008
	*sul*II	25.7	12.0	54.8	0.0001
*tet*C	*flo*R	17.8	3.9	80.8	0.0002
	*tet*A	6.4	2.0	20.9	0.002

Only statistically significant ($P<0.004$) associations are reported

Table 8.6. Unconditional associations detected between being positive for individual resistant genes and the antimicrobial resistance genes *cat*I or *flo*R (n=207)

Response Variable	Risk Factor	OR	95% Confidence Interval		*P*-value
			Lower	Upper	
*cat*1	bla_{TEM}	7.0	2.8	17.8	0.0001
	aph(3')-Ia	8.9	3.6	22.0	0.0001
	ant(3")-Ia (*aadA1*)	56.8	12.2	266	0.0001
	*sul*I	83.0	21.3	323	0.0001
	dhfrI	214	46.3	989	0.0001
*flo*R	*tet*C	17.4	3.3	92.4	0.0008
	*dhfr*XII	39.4	7.8	200	0.0001

Only statistically significant ($P<0.004$) associations are reported

Table 8.7. Unconditional associations detected between being positive for individual resistant genes and the antimicrobial resistance genes *sul*I and *sul*II (n=207)

Response Variable	Risk Factor	OR	95% Confidence Interval		*P*-value
			Lower	Upper	
*sul*I	bla_{TEM}	5.0	1.9	13.4	0.0012
	*cat*1	96.9	23.4	401	0.0001
	aph(3')-Ia	6.2	2.4	16.4	0.0002
	ant(3")-Ia (*aadA1*)	79.3	14.3	441	0.0001
	*tet*A	16.1	5.4	47.9	0.0001
	dhfrI	27.4	9.9	75.4	0.0001
*sul*II	*aph*(3')-Ia	16.5	3.1	87.2	0.0009
	*tet*B	33.9	15.5	74.3	0.0001

Only statistically significant ($P<0.004$) associations are reported

Table 8.8. Unconditional associations detected between being positive for individual resistant genes and the antimicrobial resistance gene *dhfr*I and *dhfr*XII(n=207)

			95% Confidence Interval		
Response Variable	Risk Factor	OR	Lower	Upper	*P*-value
dhfrI	bla_{TEM}	13.2	4.5	39.1	0.0001
	*cat*1	193	44.6	836	0.0001
	aph(3')-Ia	16.5	6.6	40.9	0.0001
	ant(3")-Ia (*aadA1*)	86.4	26.3	283	0.0001
	*sul*1	23.2	8.4	63.7	0.0001
*dhfr*XII	*flo*R	32.4	6.5	162	0.0001

Only statistically significant ($P<0.004$) associations are reported

Table 8.9. Unconditional associations detected between being positive for individual resistant genes and the antimicrobial resistance gene *bla*$_{TEM}$ (n=207)

Response Variable	Risk Factor	OR	95% Confidence Interval		*P*-value
			Lower	Upper	
bla$_{TEM}$	*cat*1	7.2	2.7	18.9	0.0001
	aph(3')-Ia	22.9	6.7	77.7	0.0001
	ant(3")-Ia (*aadA1*)	12.3	5.1	29.7	0.0001
	*sul*I	4.8	1.8	12.4	0.0013
	dhfrI	14.6	4.7	44.9	0.0001

Only statistically significant ($P<0.004$) associations are reported

Table 8.10. Associations between individual resistance genes summarized at the antimicrobial family level

	Aminoglycoside gene +	Tetracycline gene +	Phenicol gene +	Sulphonamide gene +	Trimethoprim gene +	Ampicillin gene +
Aminoglycoside Gene+	+	+	+	+	+	+
Tetracylcine Gene +	+	+	+	+	N	N
Phenicol gene +	+	+	N	+	+	+
Sulphonamide gene +	+	+	+	N	+	+
Trimethoprim gene +	+	N	+	+	N	+
Ampicillin gene +	+	N	+	+	+	N

+ indicates association detected
N= indicates no association detected

CHAPTER 9
ANTIMICROBIAL RESISTANCE AND VIRULENCE FACTORS IN GENERIC *ESCHERICHIA COLI* ISOLATES FROM WESTERN CANADIAN COW-CALF HERDS

9.1. Introduction

Shiga toxin or verotoxin producing *Escherichia coli* (STEC/VTEC) are the most important recently emerged groups of foodborne pathogens (Remis et al., 1984, Karmali, 1989, Beutin et al., 1998, Paton and Paton, 1998, Beutin et al., 2002, Blanco et al., 2004, Mora et al., 2004). Major STEC associated outbreaks have been experienced in Canada, Japan, the United Kingdom, and the USA (Karmali, M., 1989, Beutin et al., 1998, Paton and Paton, 1998, Willshaw, et al., 2001, Beutin et al., 2002). In people, these infections are associated with gastroenteritis that may be complicated by hemorrhagic colitis (HC) or hemolytic-uremic syndrome (HUS). Hemolytic uremic syndrome is a major cause of renal failure in children (Mora et al., 2004).

Shiga-toxigenic *E. coli* produce either one or two cytotoxins called Shiga toxins (*stx*1 and *stx*2) or verotoxins (*vt*1 and *vt*2) (Paton and Paton, 1998). Intimin is another virulence factor responsible for intimate attachment of STEC. It is encoded by chromosomal gene *eae* which is part of a large cluster of virulence genes on a pathogenicity island termed the locus for enterocyte effacement (LEE) (Kaper et al.,

1998). Shiga-toxigenic *E. coli* carrying the *eae* gene have been closely associated with HC and HUS (Karmali, 1989).

Antimicrobials are not commonly recommended for therapy of STEC infections because they can lyse cell walls leading to the release of the toxins (Waterspiel et al., 1992, Wong et al., 2000). Additionally, antimicrobials are avoided because they can cause increased expression of the toxins *in vivo* (Zhang et al., 2000). Despite the limited use of antimicrobials to treat STEC infections, there have been recent reports suggesting that antimicrobial resistance (AMR) of STEC is increasing (Gonzalez et al., 1989, Farina et al., 1996, Meng et al., 1998, Galland et al., 2001, Willshaw et al., 2001, Schroeder et al., 2002).

Virulence genes are either located in chromosomal gene clusters (pathogenicity islands) or harbored in mobile accessory genetic elements such as plasmids and phages (Groismann, 1996, Finlay and Falkow, 1997, Hacker et al., 1997). Resistance genes are also often associated with mobile DNA such as plasmids, transposons, and integrons (Jacoby, 1994, Tenover and Rasheed, 1998). Since AMR and virulence genes are carried in a similar fashion it is possible that they could be linked and then co-selected (Martinez and Baquero, 2002). Reported increases in AMR STEC isolates are of concern because antimicrobial use (AMU) could potentially enhance the selection of bacteria carrying virulence genes; ultimately accelerating the spread of virulence genes within bacterial populations (Boerlin et al., 2005).

Cattle, sheep, and goats have been implicated as the primary reservoirs for STEC (Blanco et al., 2001, 2003, 2004). AMR bacteria from livestock and farms pose a risk to public health through direct contact with livestock or production environments, and also through AMR food borne pathogens (van den Bogaard and Stobberingh, 2000, White et al., 2001). Non-pathogenic *E. coli* are also a potential problem because they can provide a pool of transferable resistance genes (Schmieger and Schicklmaier, 1999, Winokur et al., 2001).

The current literature contains several articles describing AMR in STEC's from a variety of animal populations (Gonzalez and Blanco, 1989, Galland et al., 2001, Zhao et al., 2001, Maidhof et al., 2002, Schroeder et al., 2002, Betteleheim et al., 2003, Mora et al., 2005). However, the current literature describing AMR in STEC does not always contain directly comparable information on the nature and extent of resistance in non-STEC populations. The presence of AMR within a STEC positive isolate alone does not indicate whether or not STEC are more or less likely to be resistant to antimicrobials than non-STEC organisms, or conversely whether organisms that are resistant to antimicrobials are more or less likely to contain virulence genes than organisms that are susceptible. The primary objective of this study was to investigate if presence of AMR or the presence of AMR genes is associated with the presence of the virulence genes *stx*1, *stx*2, and *eae* in *E. coli* isolates from cow-calf herds.

9.2. Materials and methods

9.2.1. General aspects of the study and sample collection

Fecal samples were collected from 480 individually identified beef calves on 91 privately owned cow-calf farms in Alberta and Saskatchewan that were accessible in the calving and nursery area (Figure 9.1). The farms were part of a larger survey for risk factors affecting calf health. Fecal samples were obtained either directly from the rectum or from the ground immediately after defecation. A separate disposable glove and container were used between samples. The majority of samples were collected from March until the end of May with a few herds having samples collected in June and early July.

9.2.2. Laboratory methods

9.2.2.1 *Escherichia coli* culture

Fecal samples were sent on ice to a diagnostic laboratory (Prairie Diagnostic Services, Saskatoon, Saskatchewan) for culture. The samples were cultured onto MacConkey agar plates at 37°C for 18 hours for isolation of *E. coli*. Each sample had at least three individual lactose fermenting colonies identified as *E. coli* using standard biochemical tests: indole, Triple Sugar Iron (TSI) slant, citrate and urea. If both dry and mucoid colonies were detected within a sample, then three isolates from each colony type were tested. In the case of one colony type, three isolates from that type were tested. Individual *E. coli* isolates were stored in 50% glycerol and Luria-Bertani (LB) broth at -80ºC until sensitivity testing was performed.

9.2.2.2 Selection of isolates for further testing

A total of 1677 isolates were identified and saved for further testing. Isolates were divided into sensitive and resistant based on the criteria listed under "Antimicrobial susceptibility testing"; 94 isolates resistant to at least one antimicrobial and 12 susceptible isolates were randomly selected using a random number generator. This subset of 106 isolates was tested for the presence of resistance genes and virulence factors. No isolate replicates from the same fecal sample were included; therefore, this sub-set of isolates represents 106 animals from 57 farms.

9.2.2.3. Susceptibility testing methodology

E. coli isolates were tested for susceptibility (Alberta Agriculture, Food and Rural Development) using a microbroth dilution technique (Sensititre®, TREK Diagnostic Systems Inc., Cleveland, Ohio) and the standard 2002 National Antimicrobial Resistance Monitoring System (NARMS) CMV7CNCD gram negative public health panel

Minimum inhibitory concentrations (MICs) for 16 antimicrobials were assessed for 106 isolates (Figure 9.2). Breakpoints for susceptibility were used, as defined by the National Committee on Clinical Laboratory Standards (NCCLS 2000) (Figure 9.2). All isolates identified with intermediate susceptibility were classified as susceptible for the statistical analysis. Amikacin results > 4µg/mL were labeled not interpretable because

the breakpoint is 4 dilutions beyond the range of the panel. The breakpoint used for streptomycin was 64μg/ml (CIPARS, 2006).

9.2.2.4. Molecular testing methodology

9.2.2.4.1. Bacterial strains and growth conditions

DNA hybridization and PCR were used to test for 24 resistance genes from 6 antimicrobial families (Département de Pathologie et Microbiologie, Faculté de Médecine Vétérinaire, Université de Montréal, Saint-Hyacinthe, Québec). The antimicrobial family, the genetic marker along with the PCR primer sequence, and source of DNA are summarized in Table 9.1.

The 28 strains used as positive controls and templates for DNA amplification were obtained from different laboratories (Maynard et al., 2003, Maynard et al., 2004). These strains were stored at -80°C in tryptic soy broth medium containing 10% glycerol (vol/vol) and were propagated on Luria-Bertani broth or agar containing one of the following antimicrobial agents at the appropriate concentrations: ampicillin (50 μg/ml), gentamicin (30 μg/ml), kanamycin (50 μg/ml), tetracycline (10 μg/ml), chloramphenicol (10 μg/ml), trimethoprim (10 μg/ml), and sulfamethazine (200 μg/ml).

9.2.2.4.2. Detection of antimicrobial resistance genes

Oligonucleotide primers for PCR amplification of antimicrobial resistance gene sequences are described in Maynard et al. (2003, 2004). Template DNA was prepared from bacterial cultures by the boiling method of Daigle et al. (1994). PCR reactions (total volume, 50 µl) contained 1x PCR buffer (10 mM Tris-HCl pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$) (Amersham Pharmacia Biotech Inc., Piscataway, N.J.), 200 µM each of the four deoxynucleoside triphosphates, 1 U of Taq DNA polymerase (Amersham Pharmacia Biotech Inc.), 25 pmol of each primer and 5 µL of template. DNA amplification was carried out in a GeneAmp PCR system 9700 (Perkin-Elmer, Foster City, Calif.) with the following conditions: 5 min at 94°C, followed by 30 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 1.5 min. An aliquot (3 µL) of each PCR reaction was resolved in a 1.2% agarose gel to confirm product size and purity. PCR products were labeled with [α-^{32}P] dCTP by using Ready-To-Go DNA Labeling Beads (Amersham Pharmacia Biotech Inc.). Colony hybridizations were performed as described previously (Harel et al., 1991).

9.2.2.4.3. Detection of virulence factor genes

Isolates were provided to a commercial diagnostic lab for virulence factor detection (Prairie Diagnostic Services, Saskatoon, Saskatchewan). Two to four colonies of each *E. coli* isolate were randomly selected from blood agar plates and resuspended in 400 µl of D-Solution (4 M guanidine isothiocyanate, 25mM Na citrate at pH 8.0, 0.5%

sarcosyl, 0.1 M β-mercaptoehanol) (Sigma Aldrich Corporate Office St. Louis, Missouri, USA). TE-saturated phenol (100 µl) (Sigma Aldrich Corporate Office St. Louis, Missouri, USA) and 100 µl of chloroform were added to each tube, followed by mixing, incubation at -20°C for 10 minutes, centrifugation for 5 minutes at 4°C and 15,000 x g and removal of the aqueous layer to a fresh tube. Phenol:chlorofom extractions were repeated until the interface was clear. Nucleic acids were precipitated by the addition of 500 µl of 95% salted ethanol (VWR International Inc. West Chester, Pennsylvania, USA) followed by incubation at -20°C and pelleted by centrifugation at 15,000 x g for 15 min at 4°C. DNA pellets were dried for 5-10 minutes between 30 and 35°C and dissolved in 80 to 100 µl of sterile water.

Oligonucleotide primers used for the PCR detection of virulence associated genes are shown in Table 9.2. PCR reactions (50 µl total volume) contained 1x PCR buffer (Fermentas International Inc., Burlington, Ontario, Canada), 2mM $MgCl_2$, 250 mM of each of the four dNTPs, 2.5 U *Taq* DNA polymerase (Fermentas International Inc., Burlington, Ontario, Canada), four primers (2 µl / primer, 20 pmol/µl) and 2 µl of template DNA. The thermocycler protocol consisted of 2 min at 94°C followed by 35 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds and a final extension at 72°C for 10 min. PCR products were visualized following electrophoresis on a 1.25% agarose gel.

9.2.5. Statistical analysis

Descriptive analyses were completed using commercially available software (SPSS 11.0 for Windows, SPSS Inc., Chicago, Illinois). All isolates were coded for the presence or absence of each resistance phenotype, resistance gene, or virulence gene considered in the analysis (Table 9.3). Summary categories for being positive for any virulence factor, for resistance to any antimicrobial, for any resistance gene, for resistance to multiple antimicrobials, or for multiple resistance genes were established.

Minimum inhibitory concentration results were classified according to the Veterinary Drug Directorate (VDD), Health Canada Guidelines (CIPARS, 2006). To facilitate consistent comparisons with the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) the same nomenclature for patterns of resistance were used (CIPARS, 2006). Multiple resistance was defined as resistance to ≥2 antimicrobials.

Unconditional associations for being positive for either phenotypic or genotypic AMR and the presence of the three virulence factors of interested were investigated using generalized estimating equations (GEE) to account for clustering within herd (SAS v.8.2 for Windows (PROC GENMOD); SAS Institute, Cary, North Carolina, USA). Model specifications included a binomial distribution, logit link function, repeated statement with subject equal to herd, and an exchangeable correlation structure. All variables investigated are listed in Table 9.3.

Multiple comparisons were accounted for using a Bonferroni correction to provide a conservative estimate for the level of statistical significance (Dohoo et al., 2003) An association was significant if $P<0.003$ after correction for 14 comparisons ($P < 0.05/k$, k = number of comparisons) (Dohoo et al., 2003).

Because no statistically significant associations were identified, post hoc power was estimated to determine whether the sample size was adequate to detect an important association between tetracycline resistance and the presence of the *stx2* and *eae* in these 106 isolates (EPI Info 6 ver 6.04d, CDC, USA, Fleiss et al., 2003).

9.3. Results

9.3.1. Description of sample population examined in AMR and virulence study

The median age of calves sampled for this study was 6 days with a range of 1 to 120 days and the dams for these calves ranged from 2 to 13 years with a median of 5 years. Of the 106 calves sampled, 58% (61/106) were male and 92% (98/106) were healthy.

9.3.2. Phenotypic antimicrobial susceptibility in the selected isolates

Ranges of observed minimum inhibitory concentrations (MICs) for the 106 isolates selected for study are summarized for each antimicrobial in Figure 9.2. The individual antimicrobials to which resistance was most commonly observed were tetracycline, sulphamethoxazole, and streptomycin (Table 9.4). Twenty-seven different resistance

patterns were represented in this sample. One isolate was resistant to 10 different antimicrobials. The most common AMR patterns detected included a grouping of streptomycin, sulphamethoxazole, and tetracycline (n=28, 27%) while the second most common grouping was sulphamethoxazole and tetracycline (n=14, 13%).

9.3.3. Resistance Genes

The most common resistance patterns detected phenotypically were also detected genotypically. Tetracycline resistance gene *tet*B, sulphonamide resistance gene *sul*II and streptomycin/spectinomycin gene *aada*1 were the most common resistance genes detected (Table 9.5). A total of 35 different multiple resistance gene combinations were identified. For multi-resistance gene isolates, the median number of resistance genes in the observed patterns was 3 with a maximum of 11. The most common resistance gene combinations detected included both sulphonamides (*sul*II) and tetracycline (*tet*B) (n=30). The next most common pattern (7 isolates) contained a grouping of *aph(3')*-Ia, *ant* (3")Ia (*aadA1*), *tetB, cat*I*, dfr*i*, sul*I and *sul*II.

9.3.4. Shiga-toxin producing *E. coli* (STEC) virulence genes

Of the 106 isolates examined, about half contained at least one virulence gene of interest (Table 9.6). The most common virulence gene detected was *stx*2. About a quarter of isolates (26.3%) contained all three virulence factors.

9.3.5. Association between AMR and virulence factors

Approximately half of the isolates were resistant to at least one antimicrobial and were also carrying at least one of the virulence genes examined (Table 9.7). Phenotypic resistance was not significantly associated with the presence of *stx*1, *stx*2, or *eae* (P>0.003) (Table 9.8).

About half of the isolates were positive for both virulence and resistance genes (Table 9.9), but the proportion of isolates containing specific virulence and resistance genes varied between 0 and 100%.The most common virulence gene detected in isolates with at least one resistance gene or with multiple (≥2) resistance genes was *stx*2. No significant associations (P>0.003) were detected between any of the resistance genes and *stx*1, *stx*2, or *eae* (Table 9.10).

9.3.6. Post hoc assessment of study power

With the current data, a 2.5 fold difference between isolates with or with out tetracycline resistance that were *stx*2 positive, could have been detected with 95% confidence and >80% power. For *eae*, the minimum difference that could have been detected in the occurrence of *eae* positive isolates that were or were not resistant to tetracycline was 3.0 fold with 95% confidence and >80% power. The actual difference was only 1.6 and 1.5 times respectively (Table 9.7), therefore study power was not sufficient to detect a significant difference if one was present at this low level but it was sufficient to detect a moderate difference.

9.4. Discussion

The primary objective of this study was to assess the association between AMR and the occurrence of *stx*1, *stx*2 and *eae* in *E. coli* isolates collected from cow-calf herds. No significant associations were detected between any of the AMR phenotypes or genotypes and virulence factors in this population of healthy beef calves. Resistant isolates were no more likely to have STEC virulence factors than susceptible isolates.

Even though virulence genes and AMR can be transmitted in a similar fashion, and associations between certain virulence genes and AMR have been detected in isolates from swine samples (Boerlin et al., 2005), there was no significant association detected between AMR and the virulence genes examined in this study. The lack of association might be due to how these virulence and resistance genes are transmitted. Most *stx* genes are thought to be encoded on bacteriophage genomes integrated into the bacterial chromosome (O'Brien et al., 1984, Acheson et al., 1998, Neely and Freidman, 1998, Muneisa et al., 2000). During transduction, DNA from a bacteriophage is interjected into a host cell where it can lead to the production of new phage particles (Schwartz et al., 2006). Subsequent replication of phage DNA and repackaging into new phage particles ultimately leads to the ability of these particles to be released (Schwartz et al., 2006). The primary limitations of transduction are the amount of DNA that can be incorporated into a phage head and the requirement for a specific receptor for phage attachment (Schwartz et al., 2006). Specificity of bacteriophages results in this mechanism of AMR transfer being relatively unimportant (Prescott, 2000). Therefore,

the lack of association between AMR and the *stx* virulence genes is not unexpected. No significant association between the virulence gene *eae* and AMR is probably the result of *eae* being carried chromosomally (Kaper et al., 1998), while many of the resistance genes investigated are often located on plasmids (Schwartz et al., 2006).

In contrast to our findings, where resistance was detected in equal proportions of STEC positive and STEC negative isolates, Bettleheim et al. (2003) reported higher levels of resistance in non-STEC than in STEC isolates, regardless of source. The main difference between these two studies was the sampling frame. The Bettleheim (2003) study included bovine, porcine, ovine, and human samples from both healthy and diseased animals and people, with the majority of AMR in non-STECs identified in porcine, symptomatic human and healthy baby samples. The bovine samples in the Betteleheim (2003) study that were both STEC positive and resistant were collected from diagnostic samples. Sick cattle have been reported to have higher levels of resistant STEC than healthy cattle (Gonzalez and Blanco, 1989, Bettleheim et al., 2003). One possible reason for this is that sick animals are more likely to have been treated with antimicrobials, potentially creating an environment more favorable for resistant populations as a result of selection pressure. Additionally, some selective advantage might be provided to bacteria that are also carrying virulence genes. Further research is needed to assess if the association between virulence factors *stx*1*, stx*2 and *eae* and AMR are different in healthy and sick cattle.

Previous reports that primarily focus on the prevalence of AMR in STEC positive isolates (Galland et al., 2001, Zhao et al., 2001, Maidhof et al., 2002, Schroeder et al., 2002, Mora et al., 2005) cannot be directly compared to the current study because isolates were selected for this study based on AMR status. The proportion of resistant isolates does not represent the background frequency in the source population. However, the primary objective of this study was not to describe the prevalence of AMR genotypes and phenotypes, but to address the relationship between AMR and *stx*1, *stx*2, and *eae*. Post hoc power calculations suggested there was sufficient power to detect important associations between the most common resistances and STEC virulence factors in this sample.

The proportion of STEC gene positive isolates can be considered in relation to previous research since AMR and virulence are not related and, therefore, the proportion of virulence genes detected should not have been biased as a result of isolate selection. The proportion of STEC gene positive isolates is similar to what has been previously reported by researchers investigating STECs in healthy adult beef cattle in Brazil (53% STEC) (Cerqueira et al., 1999), in healthy dairy calves in Japan (46% STEC) (Koybayashi et al., 2001), and from both sick and healthy animals and people in Australia (48% STEC) (Bettleheim et al., 2003). Our results differ from studies on healthy calves in Spain (23% STEC) (Blanco et al, 1996), healthy cattle at slaughter in France (70%) (Rogerie, et al., 2001), and healthy bulls, dairy and beef cows in France (18% STEC) (Pradel et al, 2000).

The most prevalent of all of the virulence genes detected in the current study was *stx*2. Other studies have reported that *stx*2 and *eae* are more often associated with severe human disease than *stx*1 (Boerlin, et al., 1999). Further investigation into the serotypes of these isolates would be important to determine their potential impact on human health.

The STEC prevalence in cattle can be influenced by many factors including sampling and detection methods adopted (Caprioli et al., 2005), potentially accounting for the wide range of reported prevalences. Additionally, the proportion of animals shedding *stx* genes also appears to vary with other factors including, animal age, and season (Cray and Moon, 1995, Chapman et al., 1997, Hancock et al., 1997, Hancock et al., 1998, Shinagawa et al., 2000, Paiba, et al., 2003).

While the literature does contain information on the proportion of resistant STEC isolates from a variety of populations including samples from healthy and diseased cattle, sheep, pigs, people, and food (Gonzalez and Blanco, 1989, Galland et al., 2001, Zhao et al., 2001, Maidhof et al., 2002, Schroeder et al., 2002, Betteleheim et al., 2003, Mora et al., 2005), we are unaware of other work that has investigated whether there is an association between AMR, measured both phenotypically and genotypically, and the virulence genes *stx*1, *stx*2, and *eae*. This work demonstrates that AMR is not any more likely in STEC positive isolates than in STEC negative isolates in this population of health beef calves.

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Figure 9.1. Schematic of number of samples, number of isolates and number of farms for each age group of animals investigated

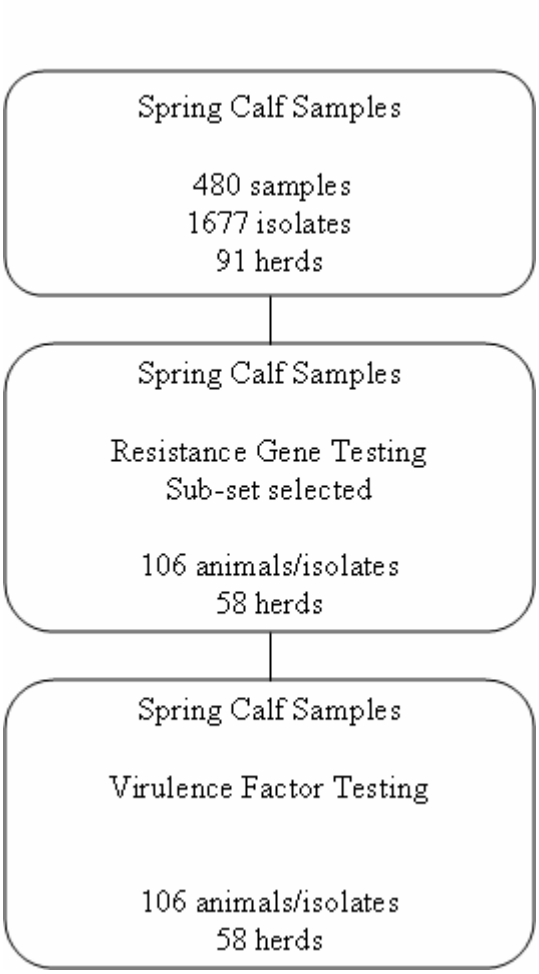

Figure 9.2. Minimum inhibitory concentration distribution for 106 isolates tested for antimicrobial sensitivity using Sensititre 2002 NARMS CMV7CNCD plate configuration. Numbers are presented as a percentage of the total isolates (n=106).

*	Antimicrobial	n	MIC Percentiles		Distribution of Isolates (%) Across Minimum Inhibitory Concentrations (MIC) Ranges																
			Median	75th	<=0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	>512
I	Ceftiofur	106	0.25	0.5				0.9	70.8	22.6	1.9	0.9	0.9	1.9							
	Ceftriaxone	106	<=0.25	0.25					94.3	2.8			1.9	0.9							
	Ciprofloxacin	106	<=0.015	<=0.015	100.0																
II	Amikacin	106	2	2							26.4	66.0	6.6	0.9							
	Amoxicillin-Clavulanic Acid	106	4	8							0.9	27.4	41.5	17.9	5.7	3.8	2.8				
	Gentamicin	106	1	1					10.4	22.6	63.2	1.9			0.9	0.9					
	Kanamycin	106	<=8	>=128										71.7				28.3			
	Nalidixic Acid	106	4	4								33.0	67.0								
	Streptomycin	106	64	64												33.0	42.5	24.5			
	Trimethoprim-Sulphamethoxazole	106	0.5	>=8				18.9	27.4	23.6	0.9			29.2							
III	Ampicillin	106	4	>=64							1.9	34.9	28.3	1.9	0.9		32.1				
	Cefoxitin	106	4	8								14.2	57.5	20.8	0.9	6.6					
	Cephalothin	106	8	16									17.0	50.0	25.5	1.9	5.7				
	Chloramphenicol	106	8	52								1.9	29.2	41.5	1.9		25.5				
	Sulphamethoxazole	106	>512	>512											17.9						82.1
	Tetracycline	106	>=64	>=64									12.3	0.9		0.9	85.8				
IV																					

Roman numerals I-IV indicate the ranking of human importance, VDD. The un-shaded fields indicate the range tested for each antimicrobial in the 2002 plate configuration. Vertical double bars indicate the breakpoints and highlighted cells locate the median. Numbers in bold font are the number of isolates with growth in all wells within the tested range, indicating the actual MIC is greater than that range of dilutions.

Table 9.1. Antimicrobial family, genetic marker, primer sequence, GenBank accession number and DNA source for resistance genes tested.

Antimicrobial family	Genetic marker	PCR primer sequence (5'-3')		Amplicon size (bp)	Positive control	Source of DNA
		Forward	Reverse			
Beta-lactams	*bla*$_{TEM}$	GAGTATTCAACATTTTCGT	ACCAATGCTTAATCAGTGA	857		R. C. Levesque
	bla$_{SHV}$	TCGCCTGTGTATTATCTCCC	CGCAGATAAATCACCACAATG	768		R. C. Levesque
Aminoglycosides	*aac(3)-Iia* (*aacC2*)	CGGAAGGCAATAACGGAG	TCGAACAGGTAGCACTGAG	740		D. Sandvang
	aac(3)-IV	GTGTGCTGCTGGTCCACAGC	AGTTGACCCAGGGCTGTCGC	627		J. Harel
	aph(3')-Ia (*aphA1*)	ATGGGCTCGCGATAATGTC	CTCACCGAGGCAGTTCCAT	600		J. Harel
	aph(3')-Iia (*aphA2*)	GAACAAGATGGATTGCACGC	GCTCTTCAGCAATATCACGG	680		J. Harel
	Ant(3")-Ia(aadaI)					
	Ant(3")-If (aada6)					
Tetracycline	*Tet*(A)	GTGAAACCCAACATACCCC	GAAGGCAAGCAGGATGTAG	888		J. Harel
	Tet(B)	CCTTATCATGCCAGTCTTGC	ACTGCCGTTTTTTCGCC	774		J. Harel
	Tet(C)	ACTTGGAGCCACTATCGAC	CTACAATCCATGCCAACCC	881		J. Harel
Phenicols	*catI*	AGTTGCTCAATGTACCTATAACC	TTGTAATTCATTAAGCATTCTGCC	547		J. Harel
	floR	CGCCGTCATTCCTCACCTTC	GATCACGGGCCACGCTGTGTC	215		D. G. White
Trimethoprim	*dhfrI*	AAGAATGGAGTTATCGGGAATG	GGGTAAAAACTGGCCTAAAATTG	391		J. Harel
	Dhfr1b					
	dhfrV	CTGCAAAAGCGAAAAACGG	AGCAATAGTTAATGTTTGAGCTAAAG	432		O. Sköld
	dhfrVII	GGTAATGGCCCTGATATCCC	TGTAGATTTGACCGCCACC	265		O. Sköld
	dhfrIX	TCTAAACATGATTGTCGCTGTC	TTGTTTTCAGTAATGGTCGGG	462		C. Wallen
	dhfrXII					
	dhfrXIII	CAGGTGAGCAGAAGATTTTT	CCTCAAAGGTTTGATGTACC	294		P. V. Adrian
	dhfrXV					
Sulphonamides	*sulI*	TTCGGCATTCTGAATCTCAC	ATGATCTAACCCTCGGTCTC	822		R. C. Levesque
	sulII	CGGCATCGTCAACATAACC	GTGTGCGGATGAAGTCAG	722		J. Harel

Table 9.2. Primer name, primer sequence, length, positive controls used and the reference for each virulence factor tested

Virulence factor	PCR primer sequence (5'-3')		Amplicon Size (bp[a])	*E. coli* + control	Reference
	Forward	Reverse			
eae	ATCTTCTGCGTACTGCGTTCA	CATTATGGAACGGCAGAGGT	790	STJ348/O157:H7	Beudry (1996)
stx1	TTAGACTTCTCGACTGCAAAG	TGTTGTACGAAATCCCCTCTG	530	STJ348/O157:H7	Woodward (1992)
stx2	CTATATCTGCGCCGGGTCTG	AGACGAAGATGGTCAAAACG	327	STJ348/O157:H7	Woodward (1992)

[a]bp =base pairs

Table 9.3. Investigation into the association between AMR phenotypes and virulence factors and between AMR resistance genes and virulence factors

Response variables	Risk factors for phenotype	Risk factors for genotype
Eae	Ampicillin	bla_{TEM}
*stx*1 and s*tx*2	Amoxicilln/Clavulanic Acid	*aph*(3')-Ia
*stx*2	Cefoxitin	*ant(3")*Ia *(aada1)*
+ for any virulence factor	Cephalothin	*tet*A, *tet*B, *tetC*
	Chloramphenicol	*catI, floR*
	Kanamycin	*dhfr*I, *dhfr*XII
	Streptomycin	*sul*1, *sul*2
	Tetracycline	AMR gene +
	Sulphamethoxazole	Multiple AMR gene + (≥2 genes)
	Trimethoprim/Sulpha	
	AMR +	
	Multiple AMR + (≥2 antimicrobials)	

Table 9.4. Prevalence of AMR phenotypes in the study samples (n=106)

Antimicrobial	% Positive (# positive / total isolates)
Amikacin	0
Amox/Clav.	6.6 (7/106)
Ampicillin	32.1 (34/106)
Cefoxitin	6.6 (7/106)
Ceftiofur	1.9 (2/106)
Ceftriaxone	0
Cephalothin	7.5 (8/106)
Gentamicin	1.9 (2/106)
Kanamycin	28.3 (30/106)
Streptomycin	67.0 (71/106)
Chloramphenicol	25.5 (27/106)
Ciprofloxacin	0
Naladixic acid	0
Sulphamethoxazole	82.1 (87/106)
Tetracycline	86.7 (92/106)
Trimethoprim/Sulpha	29.2 (31/106)
AMR +	88.7 (94/106)
Multiple AMR + (≥2 antimicrobials)	85.8 (91/106)

Table 9.5. Prevalence of AMR genotypes in the study sample (n=106)

Antimicrobial	Resistance genes	% Positive (# positive/total isolates)
Ampicillin	*bla*$_{TEM}$	32.1 (34/106)
	bla$_{SHV}$	0
Gentamicin	*aac*(3)-IV	0.9 (1/106)
	ant(2")-Ia	0
	aac(3)-Iia	0
Neomycin/Kanamycin	*aph*(3')-Ia	34.0 (36/106)
	aph(3")-Iia	0
Streptomycin/spectinomycin	*ant*(3")Ia (aadA1)	34.9 (37/106)
	ant(3")If (aadA6)	0
Tetracycline (tet)	*tet*A	17.9 (19/106)
	*tet*B	72.6 (77/106)
	*tet*C	2.8 (3/106)
Chloramphenicol	*cat*I	24.5 (26/106)
	*flo*R	4.7 (5/106)
Trimethoprim (TMP)	*dhfr*I	31.1 (33/106)
	*dhfr*Ib	1.9 (2/106)
	*dhfr*V	1.9 (2/106)
	*dhfr*VII	0.9 (1/106)
	*dhfr*IX	0
	*dhfr*XII	3.8 (4/106)
	*dhfr*XIII	0.9 (1/106)
	dhfrXV	0
Sulphonamides (sulpha)	*sul*II	25.5 (27/106)
	*sul*II	75.5 (80/106)
Gene + AMR		89.6 (95/106)
Multi-gene + (≥2 genes)		84.9 (90/106)

Table 9.6. Crude prevalence of virulence factors in the study samples and prevalence adjusted for clustering at the herd level with the 95% CI (n=106)

Virulence Factor	Prevalence (%) (# positive/total)	Predicted prevalence adjusted for clustering	95 % CI		*eae* (%)	*stx2* (%)	*stx*1and *stx*2 (%)
			Lower	Upper			
eae	17.9 (19/106)	18.0	11.3	27.3	19 (100.0)	10 (52.6)	5 (26.3)
*stx*2	41.5 (44/106)	38.9	28.9	50.0	10 (22.7)	44 (100.0)	19 (43.2)
*stx*1/*stx*2	17.9 (19/106)	17.2	10.7	26.5	5 (26.3)	19 (100.0)	19 (100.0)
Virulence factor +	50.0 (53/106)	48.1	37.7	58.7	19 (35.8)	44 (83.0)	19 (35.8)

Table 9.7. The number of isolates resistant to each antimicrobial investigated and the number (percent) of isolates resistant and positive for each virulence factor (n=106)

`	# of isolates resistant to each antimicrobial	*eae* (%)	*stx*2 (%)	*Stx*1 and *stx*2 (%)	Virulence factor +
Amikacin	0	0(0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Amox/Clav.	7	2 (28.6)	5 (71.4)	3 (42.9)	5 (71.4)
Ampicillin	34	6(17.6)	15 (44.1)	9 (26.5)	15 (44.1)
Cefoxitin	7	2 (28.6)	4 (57.1)	3 (42.9)	4 (57.1)
Ceftiofur	2	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Ceftriaxone	0	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Cephalothin	8	2 (25.0)	4 (50.0)	3 (37.5)	4 (50)
Gentamicin	2	1 (50.0)	2 (100.0)	0 (0.0)	2 (100.0)
Kanamycin	30	5 (16.7)	12 (40.0)	6 (20.0)	13 (43.3)
Streptomycin	71	15 (21.1)	32 (45.1)	14 (19.7)	38 (53.5)
Chloramphenicol	27	5 (18.5)	14 (51.9)	6 (22.2)	15 (55.6)
Ciprofloxacin	0	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Naldixic Acid	0	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Sulphamethoxazole	87	16 (18.4)	40 (46.0)	16 (18.4)	46 (52.9)
Tetracycline	92	17 (18.5)	40 (43.5)	17 (18.5)	47 (51.1)
Trimethoprim/Sulphamethoxazole	31	4 (12.9)	14 (45.2)	6 (19.4)	15 (48.4)
AMR +	94	17 (18.1)	41 (43.6)	17 (18.1)	48 (51.1)
Multi AMR + (≥2 antimicrobials)	91	91 (17.6)	40 (44.0)	16 (17.6)	46 (50.5)

Table 9.8. Unconditional associations between AMR phenotypes and virulence factors *eae*, *stx*1 and *stx*2 together, and *stx*2 (n=106)

	eae				*stx*1 and *stx*2				*stx*2			
		95% CI				95% CI				95% CI		
Antimicrobial	OR	Lower	Upper	*P*-value	OR	Lower	Upper	*P*-value	OR	Lower	Upper	*P*-value
Ampicillin	0.98	0.36	2.71	0.97	1.91	0.67	5.45	0.23	1.04	0.41	2.61	0.94
Cefoxitin	1.97	0.43	8.93	0.38	4.52	1.08	18.91	0.04	2.37	0.62	9.05	0.21
Ceftiofur	-	-	-	-	-	-	-	-	-	-	-	-
Ceftriaxone	-	-	-	-	-	-	-	-	-	-	-	-
Cephalothin	1.70	0.41	7.00	0.46	2.86	0.55	14.84	0.21	1.33	0.28	6.32	0.72
Gentamicin	-	-	-	-	-	-	-	-	-	-	-	-
Kanamycin	0.91	0.32	2.57	0.86	1.11	0.33	3.76	0.87	0.84	0.36	1.95	0.69
Streptomycin	2.36	0.89	6.28	0.09	1.28	0.37	4.47	0.70	1.39	0.62	3.11	0.43
Chloramphenicol	1.01	0.32	3.15	0.99	1.25	0.38	4.06	0.72	1.43	0.58	3.52	0.43
Sulphamethoxazole	1.41	0.42	4.69	0.57	1.14	0.32	4.12	0.84	3.10	1.11	8.70	0.03
Tetracycline	1.50	0.38	5.92	0.56	1.01	0.22	4.73	0.99	1.62	0.52	5.07	0.41
Trimethoprim/Sulpha	0.60	0.18	1.98	0.40	1.03	0.34	3.10	0.96	1.05	0.44	2.53	0.92
AMR +	1.22	0.31	4.83	0.77	0.79	0.17	3.70	0.77	1.92	0.55	6.68	0.31
Multiple AMR + (≥2 antimicrobials)	0.95	0.31	2.92	0.92	0.68	0.19	2.44	0.56	1.82	0.62	5.29	0.27

- would not converge

Table 9.9. The number of isolates positive for each resistance gene and the number (percent) of isolates positive for the resistance gene and the virulence factor (n=106)

Antimicrobial Family	Resistance Genes	Isolates positive for each gene	*Eae* (%)	*stx*2 (%)	*stx*1 and *stx*2 (%)	*positive for any virulence factor (%)*
β-lactams	bla_{TEM}	34	5 (14.7)	15 (44.1)	7(20.6)	15 (44.1)
Gentamicin	*aac*(3)-IV	1	1 (100.0)	1 (100.0)	0 (0.0)	1 (100.0)
Neomycin	*aph*(3')-Ia	36	6 (16.7)	15 (41.7)	7 (19.4)	16 (44.4)
Streptomycin	aadA1	37	5 (13.5)	19 (51.4)	9 (24.3)	20 (54.1)
Tetracycline	*tet*A	19	3 (15.8)	12 (63.2)	6 (31.6)	12 (63.2)
	*tet*B	77	15 (19.5)	31 (40.3)	13 (16.9)	37 (48.1)
	*tet*C	3	0 (0.0)	2 (66.7)	0 (0.0)	2 (66.7)
Chloramphenicol	*cat*I	26	2 (7.7)	12 (46.2)	4 (15.4)	13 (50.0)
	*flo*R	5	1 (20.0)	3 (60.0)	0 (0.0)	3 (60.0)
Trimethoprim	*dhfr*I	33	4 (12.1)	15 (45.5)	6 (18.2)	16 (48.5)
	*dhfr*Ib	2	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	*dhfr*V	2	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	*dhfr*VII	1	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	*dhfr*XII	4	2 (50.0)	2 (50.0)	1 (25.0)	2 (50.0)
	*dhfr*XIII	1	1 (100.0)	1 (100.0)	0 (0.0)	1 (100.0)
Sulphonamides	*sul*I	27	2 (7.4)	13 (48.1)	4 (14.8)	14 (51.9)
	*sul*II	80	16 (20.0)	33 (41.3)	12 (15.0)	39 (48.8)
Gene + for AMR		95	17 (17.9)	40 (42.1)	17 (17.9)	47 (49.5)
Multi-gene + for AMR (≥2 genes)		90	16 (17.8)	38 (42.2)	15 (16.7)	44 (48.9)

Table 9.10. Unconditional associations between AMR genotypes and virulence factors *eae*, *stx*1 and *stx*2 together, and *stx*2 (n=106)

	eae				*stx*1 and *stx*2				*stx*2			
		95% CI				95% CI				95% CI		
AMR gene	OR	Lower	Upper	*P*-value	OR	Lower	Upper	*P*-value	OR	Lower	Upper	*P*-value
*Bla*tem	0.39	0.25	2.09	0.55	1.14	0.40	3.24	0.81	1.06	0.47	2.39	0.89
*cat*1	0.28	0.06	1.25	0.10	0.66	0.14	3.04	0.59	1.01	0.36	2.84	0.99
*flo*R	1.05	0.09	11.9	0.97	-	-	-	-	2.33	0.29	19.0	0.43
aph(3')-Ia	0.90	0.34	2.38	0.83	1.20	0.41	3.46	0.74	0.99	0.50	1.97	0.99
*Aada*1	0.59	0.19	1.83	0.36	1.88	0.67	5.25	0.22	1.77	0.77	4.04	0.18
*tet*A	0.84	0.21	3.41	0.81	2.53	0.80	8.01	0.11	3.02	0.97	9.42	0.06
*tet*B	1.67	0.44	6.33	0.45	0.76	0.23	2.52	0.66	0.76	0.31	1.88	0.55
*tet*C	-	-	-	-	-	-	-	-	3.98	0.26	60.1	0.32
*sul*I	0.27	0.06	1.28	0.10	0.64	0.13	3.04	0.57	1.16	0.44	3.06	0.77
*sul*II	2.09	0.64	6.86	0.23	0.51	0.17	1.60	0.25	1.07	0.46	2.50	0.88
*dhfr*1	0.54	0.17	1.70	0.29	0.94	0.31	2.91	0.92	1.10	0.47	2.59	0.83
*dhfrx*II	5.02	1.05	23.9	0.04	1.49	0.25	8.74	0.66	1.61	0.32	7.99	0.56
AMR gene +	1.02	0.28	3.78	0.97	0.80	0.14	4.46	0.80	0.98	0.31	3.04	0.97

CHAPTER 10
SUMMARY AND CONCLUSIONS

10.1 Introduction

Despite the continued focus on the importance of antimicrobial resistance (AMR) and antimicrobial use (AMU) by the scientific community and the general public, there is still a need for more information. Although there have been strides in developing a greater understanding of this complex subject, especially with the introduction of molecular techniques, there are still many unanswered questions. In an attempt to fill in some of the gaps surrounding the issue of AMR in agriculture, a study of AMR and AMU in western Canadian cow-calf herds was launched. This investigation had three primary hypotheses. First, that AMR would be infrequently detected in fecal generic *Escherichia coli* isolates from cow-calf herds because these animals are extensively managed relative to most other livestock commodities. Second, routine AMU is uncommon in most cow-calf operations and that AMR would be associated with AMU. Third, associations between AMR genes would support evidence of co-selection of unrelated resistance genes and virulence factors. Specific objectives were then designed to address these questions.

The study was successful in fulfilling its objectives; specifically it describes prevalence and patterns of AMR in the different age groups commonly found on cow-

calf farms, it identifies some of the risk factors associated with AMR development in calves, it describes common reasons for treatment and the types of antimicrobials used on cow-calf farms, it provides an initial description of the relationships between AMR phenotype and AMR genes, between AMR genes, and between AMR and specific virulence factors.

10.2 Summary of highlights from each chapter

10.2.1. Antimicrobial use study

This AMU study provides some of the first documentation of AMU and reason for treatment in extensively managed cow-calf herds during calving season. At least 86% of the herds treated one or more calves or cows during the study period; however, the overall proportion of both calves and cows reported as treated was less than 14% for calves and 3% for cows. This relatively small proportion of treated animals is consistent with the finding that the majority of antimicrobials reported as used in cow-calf operations were for individual therapeutic use rather than prophylaxis, metaphylaxis, or growth promotion.

Although some oral antimicrobials were used; injectable formulations were the most commonly reported method of antimicrobial administration on cow-calf farms. In feed AMU was uncommon, ionophores were incorporated into the feed of cows and/or heifers on 25% (58/203) of the farms but no other in-feed AMU was reported in these

herds. This varies from poultry, feedlot or swine operations where in feed use plays a larger role in antimicrobial delivery (McEwen and Fedorka-Cray, 2002, Rajic, 2006).

The most commonly used products in cows were long acting injectable oxytetracyclines and penicillins. Injectable and oral sulphonamides, injectable florfenicol, and injectable oxytetracyclines were the primary drugs selected for treatment of calves. Of these products, oxytetracyclines, penicillins, and sulphonamides are readily available over the counter from farm supply outlets, and local feed companies as well as from the veterinarian. Ease of access and the relatively low cost of these products may be the reasons why these are the most commonly used antimicrobials in cow-calf herds. Alternatively, some producers reported the use of antimicrobials not specifically formulated or registered for use in cattle and/or the administration of drugs registered for use in cattle for purposes other than that for which they were registered.

Calves were more likely to be reported as treated than cows and heifers (13.5% vs 2.7%), and the primary reason reported for calf treatment was diarrhea. When considering risk factors associated with whether a calf was ever treated, male calves were more likely to be treated than female calves, and calves for which manipulation or traction was applied during calving were more likely to be reported as treated than calves that did not require assistance. The odds of calf treatment also increased with increased reports of cow treatment in the same herd.

In the cows and bred heifers the primary reported reason for treatment from January to June was metritis followed closely by interdigital necrobacillosis. Cows and bred heifers were more likely to be treated if they were assisted at the time of calving, or if they experienced post calving problems such as uterine prolapse, retained fetal membranes, or metritis. Cows and bred heifers for which manipulation, traction, or caesarian section were reported at calving were more likely to be treated than cows and bred heifers that did not require assistance at calving.

10.2.2. Prevalence study

In order to study the prevalence of AMR in cattle from western Canadian cow-calf herds, fecal samples were collected from calves in the spring and fall of 2002, from cows in the spring of 2002, and from cow-calf pairs in the spring of 2003. *E. coli* was used as an indicator organism. Resistance to drugs classified as very important to human medicine by the Veterinary Drug Directorate were infrequent in all age classes at all sampling points. From this sampling frame it was apparent that, while younger animals were much more likely to be positive for AMR, isolates from all age classes demonstrated the most resistance to the same group of antimicrobials. The data also indicated that the cow-calf pair relationship was not an important factor in transfer of AMR.

Resistance to most of the newer generation antimicrobials was infrequent. Resistances to drugs that are classified as very important in human medicine, by the Veterinary Drug Directorate, Health Canada, were detected in less than 1% of the

isolates. Additionally for the majority of drugs tested, the median MICs were well below the breakpoint for resistance for all age groups of animals investigated. Median MICs that are several dilutions below the breakpoint indicate that at the time of testing, most of the *E. coli* population in these animals were highly sensitive to those particular drugs.

Increased prevalence of AMR in young calves has also been described by other researchers (Brophy et al., 1977, Hinton et al., 1984, Hinton, 1985, Mathew et al., 1999, Khachatryan et al., 2004). The prevalence of AMR differed significantly among the age groups sampled. When comparing the prevalence of resistance to any antimicrobial, young calves sampled in the spring of 2002 were almost 10 times more likely to have AMR isolates than older calves sampled in the fall of the year. A comparable trend was detected for the cow and young calf data. Young calves were 7 to 10 times more likely to be resistant to any antimicrobial than were cows. However, there was no difference detected in AMR prevalence between cows sampled in the spring 2002 and calves sampled in the fall of 2002.

Despite differences in the magnitude of AMR in the different age groups, the three most common resistances detected were to tetracycline, sulphamethoxazole, and streptomycin. This is similar to what others have reported for *E. coli* isolates collected from dairy cattle as well as for a variety of other animal species (Kijima-Tanaka et al., 2003, Bywater et al., 2004, Khachatryan et al., 2004). For all other drugs tested, isolates had varying degrees of resistance depending on the age group from which they were

collected. Despite the ban of chloramphenicol use in livestock in Canada, chloramphenicol resistance was still consistently detected in all of the age groups examined.

The cow-calf pair relationship was not a significant determinant of transmission of resistance to the calf (P=0.36). AMR was detected in both the cow and the calf in only 5 (4.8%) pairs, of the 105 examined. While the pair relationship did not seem to be an important determinant of AMR in the calf population the presence of AMR in the cow herd was associated with AMR in the calves. For the herds that had both cow and calf samples collected in the spring of 2002, the odds that that calves would be resistant to sulphamethoxazole or tetracycline increased with the proportion of cows that were resistant to sulphamethoxazole (OR, 7.5, P=0.02) or to tetracycline (OR, 6.1; P=0.01) respectively. These findings indicate that the individual cow is not the primary determinant of the AMR status of her calf, but that the frequency of common types of resistance in the calves is associated with exposure from the cow herd or from contamination of the environment by the cow herd.

10.2.3. Risk factor study

Vaccination practices and the use of most antimicrobials in these herds was not significantly associated with the frequency of AMR in commensal *E. coli* isolated from calves. Herd use of sulbactum:ampicillin and gentamicin were, however, identified as risk factors for the incidence of antimicrobial resistance for several unrelated

antimicrobials. These findings suggested the need to explore the potential importance of co-selection at the molecular level in these isolates.

10.2.4. Molecular studies

Assessment AMR at the genetic level is an important tool in the understanding and the control of AMR (Lanz et al., 2003). This study demonstrated the importance of characterizing resistance in generic *E. coli* using both phenotypic and genotypic methods. It is apparent that the relationships between phenotypes and resistance genes are extremely complicated. The extensive number of relationships between individual AMR phenotypes or specific resistance patterns and individual resistance genes or gene families suggests that there must be extensive linkage, and that there is a high probability of co-selection when one type of resistance is perpetuated. The type of linkages may be secondary to bringing attention to the message that AMR selection is not an independent process, but that there are numerous associations between resistance to individual antimicrobials and resistance genes and among resistance genes.

In addition to the expected associations between phenotypic resistance to specific antimicrobials and their respective resistance genes, numerous other associations were detected. Some of the strongest associations were observed between ceftiofur and *flo*R chloramphenicol and *dhfr*I, trimethoprim sulfamethoxazole and *cat*I, and tetracycline and *sul*II. Each type of phenotypic resistance examined was associated with genetic resistance to an average of five families of antimicrobials. Phenotypic resistance to streptomycin, tetracycline, and sulphamethoxazole were each associated with the

presence of resistance genes from all six of the families of antimicrobials examined in this study. The strong association between phenotypic resistance and resistance genes from different families of antimicrobials may indicate gene linkage.

The complex nature of AMR was also demonstrated by the large number of associations of moderate to substantial magnitude that were detected between resistance genes. Some of the strongest associations were between *tet*B and *sul*II, *sul*I and *cat*I, *sul*I and *aadA*1, *aadA*1 and *dhfr*I, and *cat*I and *dhfr*I. The streptomycin gene, *aadA*1, was significantly associated with at least one gene from all six families of antimicrobials investigated. S*ul*II and *tet*B were strongly associated with each other, while *sul*I was strongly associated with both *cat*1 and *aadA*1. These associations may help indicate why certain phenotypic resistance patterns are seen within commensal *E. coli* and may warrant further molecular studies.

10.2.5. Virulence and AMR

The primary objective of this study was to assess the association between AMR measured by the presence both phenotype and genotype and the occurrence of *stx*1, *stx*2 and *eae* in *E. coli* isolates collected from cow-calf herds in Western Canada. No significant associations were detected between any of the antimicrobial resistant phenotypes or genotypes and the virulence factors of interest. Resistant isolates were no more likely to have STEC virulence factors than sensitive isolates.

10.3. Study limitations

A review of the limitations of this project suggested some areas where changes might be considered for future studies in cow-calf herds. The first limitation was that herd and individual animal selection was not random. Enrolled herds were a volunteer, convenience sub-set of herds already participating in a larger health and productivity study. Individual animal samples collected were also convenience samples. The lack of random selection for both herds and individual animals could result in selection bias and potentially affect the generalizability of the study. However, participant cooperation was necessary for the collection of quality data. The logistics of sampling specific animals, given the need for additional animal handling and significant time contributions from the producers, would not have been tolerated by the herd owners during calving season. For these reasons it would be difficult to implement a true random selection of either the herds or individual animals without considerable additional resources for personnel and incentives to the participants.

From the Agriculture census in 2001, Statistics Canada reported that the average beef size for Canada was 53. In Saskatchewan and Alberta the average herd sizes were 58 and 74 beef cows per herd with 15 and 20 replacement heifers respectively. The average study herd size of 180 is larger than that reported by Statistics Canada. Because herds were enrolled in the productivity study based on their ability to provide the required data, these herds probably represent some of the more progressive, commercially viable, and intensively managed herds in western Canada. The herds providing data for this thesis therefore represent the prevalence of AMR and AMU in

this sector of the industry. The prevalence of AMR and AMU may be different in the few very large cow-calf herds that receive little or no treatment interventions or the very small herds present on some mixed or hobby farms.

The initial AMU data collected from the cow-calf herds in this study were limited. First, only treatment records from January until June were included in the analysis. The reason for limiting the records to this time frame was that this period covers the months that the herds would have been relatively confined and under observation for calving. The AMU records during this period were, therefore, more likely to be accurate and complete than records outside of this time frame. The time around calving also represents the period when the majority of AMU occurs in cow-calf herds and potentially the highest risk period for the development of AMR. The information reported can not be directly extrapolated outside the study period because risks and treatment practices differ at other times in the production cycle. For example, animals are much less likely to be treated for infectious bovine keratoconjunctivitis (pinkeye) during the winter months than during the summer and are very unlikely to be treated for metritis or other calving complications while on summer pasture.

Although individual animal records were available for this period, details on the specific antimicrobials used and the dose given were not reported by most herd owners. The data collection forms were designed for a separate study and were not intended to be used for this purpose. The lack of specific AMU data precluded investigation into the relationship between specific antimicrobials and resistances in individual animals. This

limitation could be over come in future cow-calf studies with the careful design of treatment data capture instruments.

Under-reporting is another potential issue in AMU data collection. In this project under-reporting of individual animal treatments was unknown, but data collection personnel estimated at least 20% of participating herd owners did not consistently complete individual treatment records. Under reporting could have resulted in misclassification of some treated animals as not treated. This would likely have biased the association between individual animal treatment and AMR towards the null. To supplement the individual treatment record information, an attempt was made to collect additional data on the type of antimicrobials used by administering a questionnaire at the end of calving season where producers reported the frequency of use of specific products for each herd. However, retrospective data collection as done here has the potential for recall bias.

The risk factor paper was also limited by the manner in which the AMU data were collected. Because the proportion of calves treated and AMU data were summarized for the entire period and not relative to the time of sample collection on that farm, there is also the potential for misclassification bias of exposure relative to the time the outcome of interest was measured. The herd might have been considered exposed to a certain antimicrobial or antimicrobials, but that exposure may have been subsequent to the sample collection. To assess the effect of these limitations on the study, animal

exposure could be measured through individual treatment records that included the type of antimicrobial used, the date of use, and potentially the volume.

The molecular aspects of this work were intended as an initial exploration of the utility of these methods to examining the question of co-selection in cow-calf herds. This part of the project was not intended to be exhaustive or to provide definitive answers on this problem. Isolate selection was based on resistance status to maximize study power to address association between phenotypic and genotypic resistance. The proportion of resistant isolates therefore does not represent the background frequency in the source population.

The second limitation of the genetic study was that the diagnostic lab did not test for integrons or extended spectrum beta-lactamases. The latter would have been informative in light of findings by Read et al. (2005) in a recent feedlot study. Read et al. (2005) reported the presence of extended spectrum beta-lactamases carrying the bla_{cmy2} beta-lactamase gene. This gene was associated with therapeutic use of florfenicol, oxytetracycline or tilmicosin at entry into the feedlot.

From the genetic work and the risk factor analysis in the spring calves, co-selection of AMR appears to be a contributor to AMR patterns in cow-calf herds. Investigation into the presence of certain plasmids would therefore be useful.

Testing for hemolysin in addition to the other virulence factors would be interesting because the hemolysin virulence gene may be carried on plasmids with AMR genes. These additional molecular components should be included in any future studies of cow-calf herds.

10.4. Conclusions

Cow-calf herds are not a significant reservoir for AMR to antimicrobials classified as very important to human medicine such as ceftiofur. This is worth mentioning as cow-calf farms are the most common type of livestock operation in western Canada. Finding limited resistance to antimicrobials of very high importance in human medicine indicates that at this time cow-calf herds do not pose a significant risk to human health. However, since there can also be the co-selection of underlying AMR genes, there is the possibility of AMR genes being perpetuated despite no phenotypic evidence of resistance. Continued monitoring of both the phenotype and the underlying AMR genes would be needed to see if this pattern changes over time and with the availability of new antimicrobials.

These data indicate that young calves have the highest prevalence resistant generic fecal *E. coli* in cow-calf herds. Since young calves shed the highest percentage of resistant organisms control should consider them as a source of AMR organisms. However, it is also important to recognize that while cows may shed a lower proportion of resistant isolates they also contribute more manure to the environment than do calves; therefore, they can not be ignored in developing on farm AMR control

programs. Additionally, given the similarity in the patterns of AMR observed across age groups, and the relationship between AMR in the cow herd and AMR in the calf population the cow herd is likely a reservoir of exposure for calves to resistant organisms. In order to develop a plan for AMR control, continued research is needed to understand why AMR is higher in very young calves and how the *E. coli* population changes after the spring and during the summer pasture season prior to weaning.

Despite the limitations, this study does provide the first available documentation of the proportion of calves, heifers, and cows reported as treated during the calving season and the types of conditions most often treated for in a large number of western Canadian cow-calf herds. The study also provides some initial information about AMU practices in these herds which can be used to help address issues such as extra-label drug use, prophylactic treatment of entire calf crops, and the importance of minimizing dystocia in reducing the need for treatment of either cows or calves.

Aside from the molecular interest in understanding AMR at the gene level, we can also consider the broader implications of the extensive number of associations detected between families of antimicrobials. The relationships between resistance genes allow us to begin to comprehend the magnitude and the complexity of the epidemiology of AMR. The demonstration of this network of associations also brings into question the definition of "prudent use" and the impact of these associations on developing policy and clinical practice guidelines to minimize AMR. The implication is that current attempts to limit the emergence or spread of AMR based on careful restriction of the

choice of antimicrobials will not prevent selection for a number of unrelated AMR genes. Therefore, the assessment of AMR at the genetic level is a critical tool in the understanding and the potential control of AMR (Lanz et al., 2003).

In addition to the antimicrobial selection pressure itself there are hundreds of bacterial genera and species interacting and adapting to many variables within an animal production system and, therefore, understanding the complete effects of AMR and AMU in these production systems is extremely complex (White and McDermott, 2001). This study does provide insight into the farm-level treatment factors that can influence AMR found in cattle in cow-calf operations.

This project demonstrated the level and type of resistance encountered on cow-calf farms as well as common risk factors for the presence of AMR. It also examined reasons for AMU in these herds. These data can be used by veterinarians and producers to incorporate specific interventions designed to minimize the need for AMU and to understand how certain practices may lead to increased AMR.

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